MEASUREMENTS OF THE EXCHANGE OF CERTAIN DISSOLVED SUBSTANCES BETWEEN VOLUNTARY MUSCLES AND SALINE SOLUTIONS: AN ATTEMPT TO STUDY THE STRUCTURE OF LIVING MUSCLES BY PHYSICO-CHEMICAL MEANS.

by

Abdul Ghaffar, B.Sc., M.B.

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INTRODUCTION.

In biochemical estimations it is usual to treat a tissue as a homogeneous mass in which various chemical substances are uniformly distributed, but it is reasonable to suppose that the actual distribution of its constituents may differ considerably in different parts of its substance. In the first place the substance concerned may be insoluble in the tissue water and therefore deposited in one part of its cells. But there is considerable evidence that many soluble and diffusible substances like glucose, sodium and potassium are by no means equally distributed throughout the water of a tissue. Vapour pressure studies of Hill and Kupalov (1930) indicate that in the case of muscle, most of the water is 'free' water, that is, it is capable of dissolving soluble substances added to it. There are, however, several factors that may tend to cause a difference in the concentration of various dissolved substances in different parts of a muscle. The substance concerned may be irreversibly combined with the proteins or adsorbed on some interface of its fibres. A membrane equilibrium of some kind may keep its concentration in different parts of the muscle at different levels. Or a certain fraction of muscle water may be enclosed within membranes which do not allow an exchange of certain dissolved substances across them. It is evident that in any of these conditions the concentration of dissolved substances may vary considerably in different parts of a muscle.

Some insight into the histological chemistry of muscle is gained by a study of the diffusion of various substances from or into a muscle when immersed in a saline solution of known composition. Thus it was shown by Eggleton (1930) that urea distributes itself between the muscle and the solution in such a manner as to indicate that the whole of the water of muscle is available to dissolve urea. On the other hand, in the case of inorganic phosphate (M.G. Eggleton, 1933) and carnosine (Eggleton and Eggleton, 1933), only about 30 p.c. of muscle water seems to be concerned in the diffusion system and it appears that about 70 p.c. of the muscle water is enclosed within membranes that are impermeable in these circumstances to phosphate and carnosine.

In the present investigation the diffusion of certain dissolved substances into and out of muscle has been studied. Two of these, lactate and bicarbonate, are normally present in the animal body, while the other two — iodide and iodoacetate — are extraneous substances.

In the concluding chapter the results obtained by the writer have been compared with those of the diffusion of various substances as found by other investigators, and an attempt has been made to form an idea of the normal concentration of various dissolved substances in that portion of the muscle into which these can diffuse, and in the rest of the muscle.

Diffusion of d-lactate into and out of skeletal muscles of frog.

METHODS.

(a) Chemical.

Since sarcolactic acid is dextro - rotatory it was considered desirable to use a salt of d - lactic acid in preference to one of the racemic (fermentation) acid, as it was felt that the muscle might behave differently towards the two salts.

Lactic acid was prepared from butchers meat and separated as the zinc salt. The method of preparation and purification is given in the Appendix B;p. 88

For experimental work fresh solutions of sodium lactate were prepared by adding a slight excess of M/10 sodium phosphate solution (consisting of 4 parts of di- to one equivalent part of mono-sodium phosphate) to the requisite quantity of a solution of zinc lactate, to precipitate all zinc as zinc phosphate. Sodium d-lactate thus prepared was kept in cold storage and a preparation was used for not more than one week.

Ringer solution containing 0.71 p.c. sodium chloride was used in diffusion experiments. Lactate Ringer of required strength was prepared by replacing an equivalent amount of sodium chloride in the Ringer by sodium lactate. The pH of the solution used was 7.1, but it was not thought necessary to buffer the Ringer as it was found that for small quantities of Ringer used in the

experiments (1 - 1½ times the weight of muscle), the final pH of Ringer was always about 7.4, and this is in agreement with the observation of M. G. Eggleton (1933), who found that the pH. of Ringer fluid is ultimately about 7.4 for resting muscles whether the initial pH was 7.1 or 8.0

Lactate acid was estimated by Clausen's (1922) technique as modified by Friedemann, Cotonio and Shaffer (1927), but without aeration. The details of the apparatus used, and the consistancy of the results obtained by this method, are discussed in Appendix A., p 83.

(b) Physiological.

The experimental procedure adopted was that used by Eggleton (1930), for the determination of the concentration of creatine in muscle. The animals were killed by pithing the brain and immediately afterwards transecting at one stroke the urostyle, the iliac bones and the sciatic plexus, about one centimetre caudal to the last vertebra. Only the Gastrocnemii of R. esculenta (Dutch or Hungarian) were used. The frogs used were either fresh from the tank or those cooled previously for 1 - 2 days at 0°C. The muscles were carefully dissected and removed with as little injury as possible, and if adventitious contractions occurred the preparation was discarded. The muscles were then rapidly blotted on moistened filter paper, weighed, and one of

the pair transferred to a tube containing a weighed quantity of cooled "low lactate" Ringer's fluid, containing, say, no lactate, and the other to a "high Lactate" Ringer, containing say, 100 mg. per 100 g. of Ringer.[1]

Some preliminary experiments had shown that it takes 3 - 3½ hours contact between muscle and Ringer to reach lactate equilibrium, and there was no measurable change in the concentration of lactate in the Ringer after this period even if the immersion was continued for 24 hours[2]. Diffusion was therefore allowed to proceed for four hours in all cases. All experiments were carried out at 0° C. In the case of muscles of frogs fresh from the tank, the tubes were corked and kept in cold storage, while oxygen was bubbled through in the case of frogs previously cooled for 1 - 2 days at 0° C.

After four hours' immersion the muscles were removed, blotted and weighed and ground up in 4 p.c. previously cooled trichloracetic acid. The two samples of the Ringer's fluid were also weighed and similarly treated. Lactic acid was estimated by the usual method already mentioned, six determinations being made in each experiment, namely for 'low' and 'high' lactate Ringer before and after the experiment

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1. The results of all calculations are given in terms of lactic acid although the free acid was never present in the diffusion system
2. An account of these experiments is given in the Appendix C; p. 91.

and for the two muscles at the end of the experiment.

Some experiments were performed on fatigued muscles and on muscles in heat rigor. Fatigue was induced by stimulating the hind limbs of pithed frog with single induction shocks by inserting pin electrodes under the skin of the toes of the two feet, to ensure, as far as possible, equal accumulation of lactic acid in the muscles of the two limbs. The diffusion experiment was then carried out on the two gastrocnemii as in the case of fresh muscle, but nitrogen was bubbled through the Ringer continuously to prevent oxidative recovery. Rigor was induced by incubating the muscles at 40° C. for 5 - 6 hours, after which they were given a preliminary overnight soaking in a lactate Ringer solution. They were then removed, weighed, and immersed in 'low' or 'high' lactate-Ringer for the diffusion experiment. The advantage of the preliminary soaking is that there is little or no change of weight in the muscles during the subsequent diffusion experiment. In this case the final concentration of Ringer in which the muscle was given a preliminary soaking was also determined.

RESULTS.

The results of the diffusion experiments on cooled, fresh and rigor muscles are represented graphically in Fig.1 . in which the final lactate concentrations of Ringer solutions are plotted against the final concentrations in the muscle.[1] The line A, passing through points for fresh muscles proves to be straight, a fact which eliminates the possibility of adsorption being a significant factor in the equilibrium set up. The line cuts the vertical axis at a point representing a concentration of 60 mg. of lactic acid in the muscle, suggesting that in the fresh muscles examined, on an average about 60 mg. of lactic acid per 100 g. of muscle does not take part in the diffusion process. The slope of the line indicates that, presuming the final equilibrium to be a simple osmotic one, only 26 p.c. of the muscle is concerned in the diffusion process. Since the total water content of muscle amounts to about 80 p.c. of its total weight, it follows that only about 32 p.c. of the muscle water seems to take part in the diffusion process.

Line B for muscles from previously cooled frogs runs parallel to line A, indicating that in this case also the same percentage of muscle water takes part in the diffusion process. This line, however, runs nearer the horizontal axis than the line A and

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1. Full details of these experiments are given in Appendix E ; p 95-97.

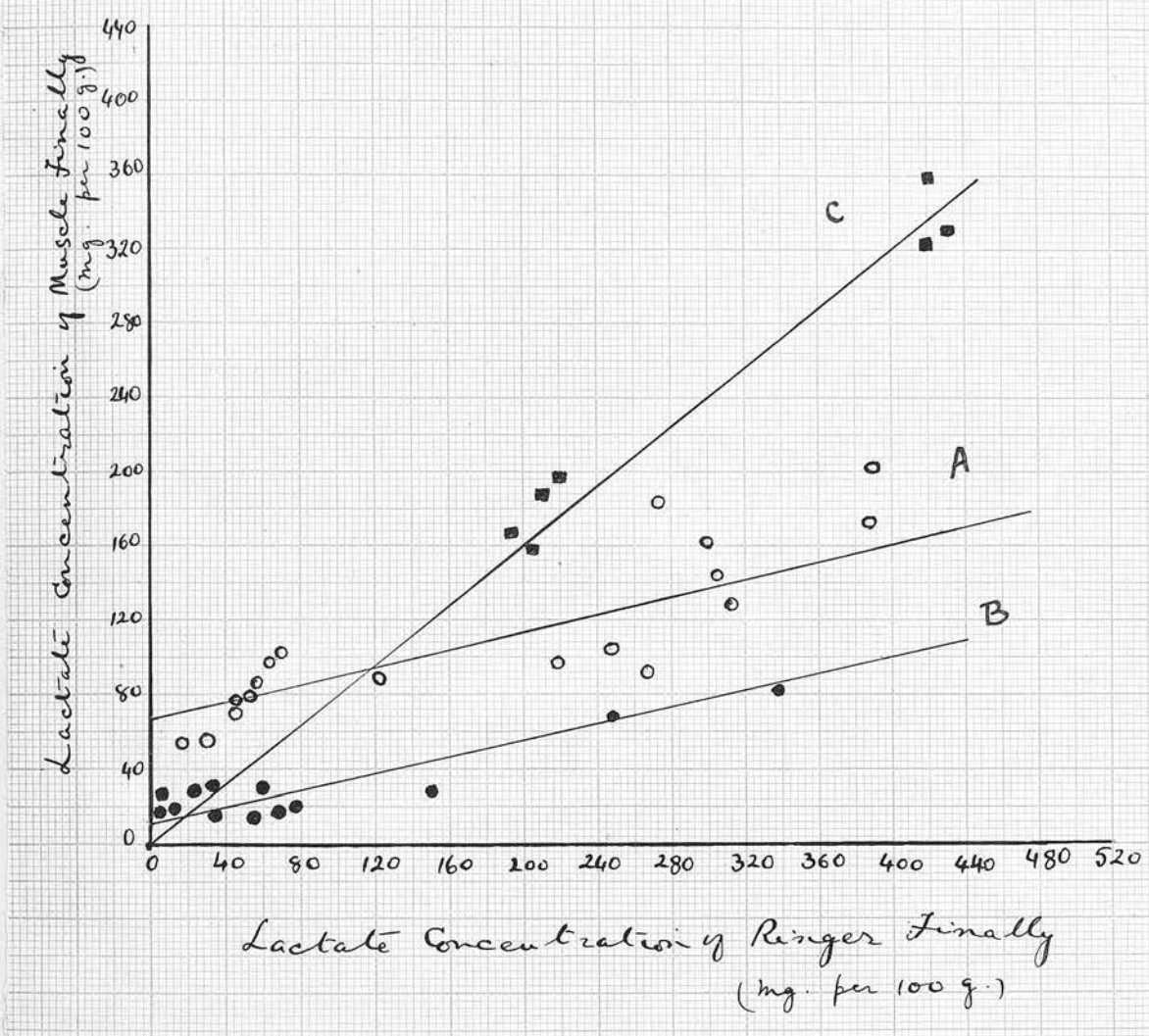

FIG. 1. Relative concentrations of lactate in Ringer's solution (containing varying amounts of sodium d-lactate), and frog muscle immersed in it after the two had reached diffusion equilibrium with regard to lactate.

A - Muscles of frogs fresh from tank.
B - Muscles of frogs cooled to 0°C for 1 to 2 days.
C - Muscles in heat rigor.

indicates an average of 10 mg. of lactic acid per 100 g. of muscle not involved in the diffusion process.

In the case of rigor muscles (line C) the line when extended passes through the zero point showing that in this case the whole of the lactate contained in the muscle takes part in the diffusion process. Further, it will be seen that the ratio of the concentrations in Ringer and muscle at equilibrium is 100 : 80. The total water content of muscles in heat rigor after preliminary soaking was found to be 83 p.c. of the muscle weight.[1] It follows that in the case of muscle in heat rigor, nearly the whole of the water of the muscle is available to take part in the diffusion process.

The proportion of the muscle water (x) taking part in the diffusion process, as well as its lactate content (y), can be more accurately determined for each pair of muscles by a simple algebraic operation from the initial and final lactate contents of the two Ringers used, as the final concentration depends on the weight and the initial lactate content of (a) Ringer used, and (b) that portion of muscle water which takes part in the diffusion process.

An example of an actual calculation may be cited. In this case the weight of the "low" and "high" lactate Ringers was 1.854 g. and 2.106 g. The

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1. For experimental details see Appendix D ; p. 93.

initial lactic acid contents of these were 0 mg. and 1.74 mg. respectively. The final lactic acid concentrations (mg. per G.) were found to be 0.096 and 0.713 respectively. The calculation then took the following form:

(1) $0.096x + 0.096 \times 1.854 = y + 0$

(2) $0.713x + 0.713 \times 2.106 = y + 1.74$

Resolving these equations, we get $x = 0.674$ g. and $y = 0.24$ mg.

From this value of x, the fraction (α) of the total muscle water involved in the diffusion is calculated, taking the total muscle water to be equal to 80 p.c. of the weight of the muscle. In the present example the muscle weighed 2;217 g. and α is therefore $\frac{0.674 \times 100}{4/5 \times 2.217} = 38$ p.c.

In the case of muscles in heat rigor the value of x and therefore α can be calculated from a single muscle experiment, as the initial concentration of lactate in x is given by the lactate concentration of the Ringer in which muscle was given preliminary soaking.

The results of these experiments thus calculated are given in table 1 . In the case of fresh and previously cooled frogs, the average value of α is about 35, which means that when the equilibrium is established between the muscle water and the Ringer, only about 35 p.c. of the muscle water appears to come into diffusion equilibrium with Ringer, so far as

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TABLE 1. Proportion of Muscle Water concerned in the exchange of lactate between Frog Muscle and Ringer Solution.[1)]

Condition of Muscle	No. of Expts.	Percentage of Muscle Water involved in the diffusion process. (α)	
		Maximum range of Variation	Mean Value
From frog fresh from the tank	9	28-48	36
From frog cooled to 0°C. for 24 hrs.	7	31-42	34
Muscles in heat rigor	7	76-101	92

1. For the calculated value of α for each experiment, see Appendix E, p. 95-97.

lactate is concerned, the rest of the water being as if shut off by membranes which do not allow lactate exchange across them. Following the Eggletons' terminology, the former may be called the water contained in the 'interspaces' and the latter that of the 'cells' of the muscle, though as explained by them, we have no evidence whether these fractions are identical with the anatomical interspaces and fibres.

The average value of α for muscles in rigor is 92 p.c., which shows that nearly the whole of the water of muscle is available for exchange of lactate with Ringer.

FATIGUED MUSCLE.

Diffusion experiments with fatigued muscles brought out the rather unexpected result that the value of α is very low, so that only an approximate value can be found for it. The result are given in Table 2.[1)]

This relative diminution in the size of 'interspaces' when the muscle is fatigued can also be deduced from the experiments of Devadatta (1933), which were designed to determine the concentration of lactate in the Ringer that is in diffusion equilibrium with the muscle lactate. The exact amount of saline used is not given in his tables, but he mentions that it was $1\frac{1}{2}$ to 2 times the weight of the muscle. On this basis it is possible to

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1. For fuller details of these experiments see Appendix E. p 98.

TABLE 2. Diffusion of lactate into and out of fatigued muscles of Frog.

	Concentration of lactic acid in Ringer (Mg. per 100g.)		Calculated Value of (α), (the percentage of Muscle Water involved in the diffusion process)	Initial concentration (Mg. per 100g.) of Lactic acid in Muscle (Calculated)
	Initial (1)	Final (2)	(3)	(4)
1. 1	0	42	9	320
L	294	319		
2. 1	0	38	7	284
L	325	339		
3. 1	0	35	-3	259
L	294	352		
4 1	0	31	2	256
L	294	324		

1 = "low lactate" Ringer.
L = " high lactate" Ringer.

Column 4 is not needed for the evaluation of α, but is included here to save space; for its significance, see p. 17.

determine approximately the value of α from the data given in his tables. It is found that the value of α varies from 25 to 75 in the case of fresh muscles, and from 4 to 24 in those that were fatigued. The wide range of variations in these values in different experiments are no doubt in great part due to the uncertain factor of the amount of saline used, but the fall in the value of α due to fatigue is obvious.

The explanation of the low value of α in the fatigued muscles probably lies in an alteration in the "interspaces" of the muscle. In fatigue the osmotic pressure inside the cells rises, consequently they swell and the 'interspaces' tend to disappear. This interpretation is supported in an indirect way by the observation of Eggleton, Eggleton and Hill (1928), that in fatigue the coefficient of diffusion of lactic acid in muscles that have been previously stimulated, falls from 6×10^{-5} for slightly stimulated to 5×10^{-6} for those fatigued to a pronounced degree. As the diffusion constant is proportional to the square of the surface area through which diffusion can take place, a diminution in the value of α will tend to lower the coefficient of diffusion of the muscle.

Relative concentration of lactate in 'interspaces' and 'cells' of frog muscle.

In calculating the value of α (the 'interspace' water as a fraction of the total), the weight of the muscle water concerned in diffusion, and the lactate

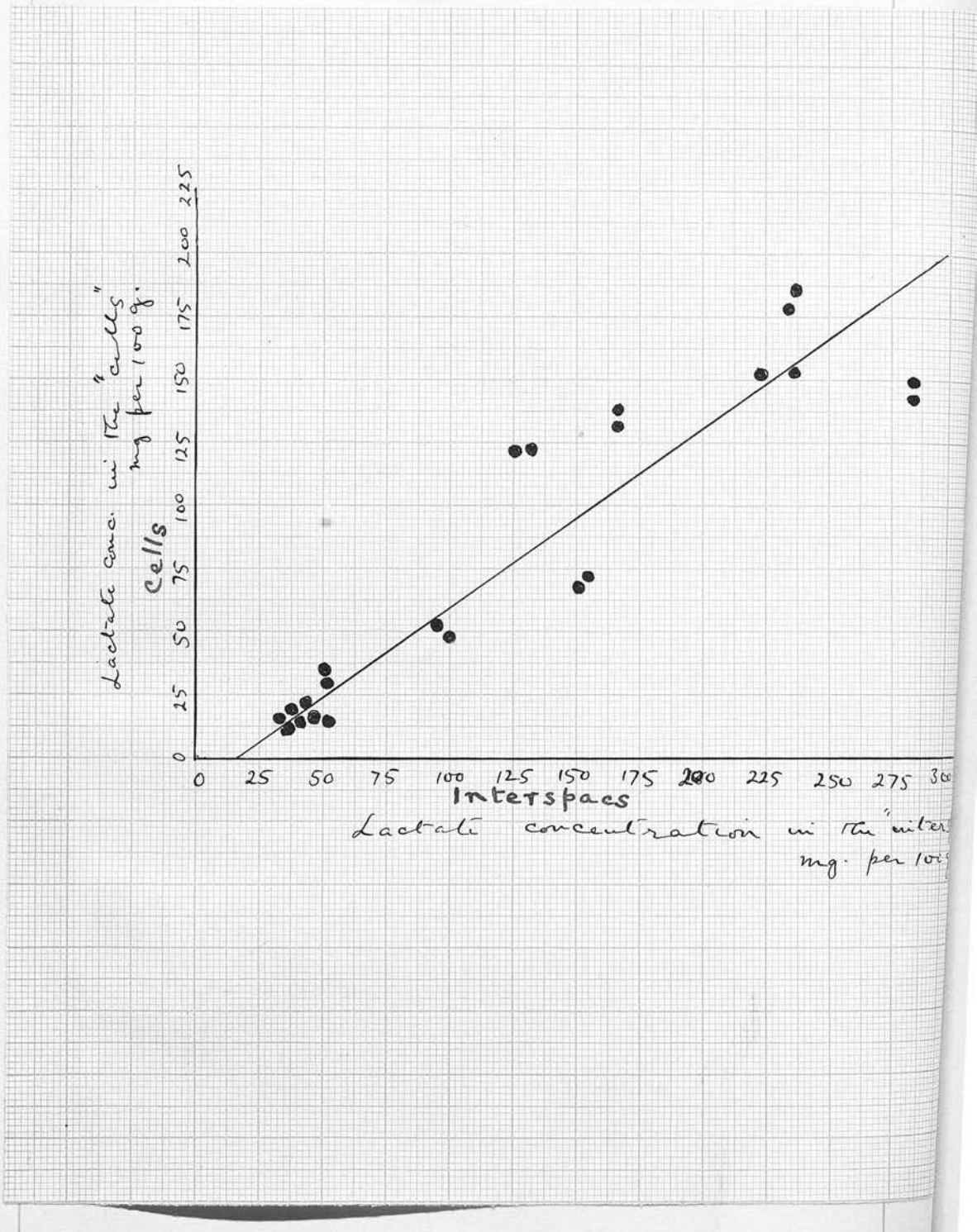

FIG. 2. Relative concentration of lactate between the "cells" and "interspaces" of the frog muscle.

For explanation, see Text.

content of this portion were determined. From these values, the concentration of lactate in this portion of muscle water can be obtained directly.

It is also possible to calculate, from the data available, the lactate concentration of the 'cells'; for in any muscle - Ringer system, the initial lactate content of the muscle is given by its measured final content corrected for the measured loss or gain in the Ringer's fluid. The lactate content and water content of the 'interspaces' are known already (see p.10). The lactate and water content of the 'cell' fraction and hence the concentration of lactate in this portion can therefore be evaluated. Lactate concentrations in "interspaces" and "cells" thus calculated in the case of fresh and cooled frogs gave the rather unexpected result that the concentration of lactate in the "interspaces" is always higher than in the "cells".[1] These results are graphically represented in Fig.2 ., in which abscissae give the concentrations in the "interspaces", and the ordinates those in the "cells". The exact relations of lactate content in the two parts will probably depend on experimental conditions, but the fact that the line passing between these points is a straight line justifies the suggestion

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1.. The possibility that a lower value of lactic acid may be due to oxidative removal during the course of the experiment was negatived by some observations which showed that there is no appreciable decrease in the overall lactic acid content of muscle - Ringer system in 4 hours under these experimental conditions.

that despite the complete independence of these two fractions in the isolated relaxed muscle, their lactate concentrations do not vary independently when the muscles are in situ. This apparently contradictory conclusion is discussed later.

In the case of fatigued muscle the value of α is too small to allow of an estimation of concentration of lactate in the two fractions of the muscle with any degree of accuracy, but there is reason to believe that in this case also the concentration in the "interspaces" is higher than in the "cells". This will be clear from the study of the figures given in Table 2., which gives the calculated results of some of the experiments on the fatigued muscles. It will be seen that in experiments 2 - 4, in each case the concentration of the total muscle lactate is lower than that of high lactate Ringer, but the diffusion experiment showed that in each case some lactate diffused from the muscle into the Ringer, so that the final concentration of lactate in the Ringer was higher than its initial figure. The conclusion is that though the total lactate concentration of muscle water was lower than the high lactate Ringer, that of the interspaces was higher. It seems that in fatigued muscle, just as in resting muscle, concentration of lactate in the "cells" is lower than in the "interspaces".

This may be the possible explanation of the observation by Eggleton and Evans (1930) who found that (in the case of dogs) in fatigue the passage of

lactate from muscle into blood continues for a considerable time after the concentration of the arterial lactate has risen higher than the muscle lactate. This is to be expected if the concentration of the "interspace" lactate is higher than the "cell" lactate, for then at a certain stage in the recovery process the lactate in the interspaces will be higher than blood lactate, though the total muscle lactate would show a lower value.

Exchange of lactate between the "cells" and the "interspaces".

The experiments on diffusion equilibrium described in this paper give results which seem to be most simply interpreted on the hypothesis that only a fraction of the water of a living muscle is concerned in the diffusion system. Interpreted thus these results suggest that two-thirds of the muscle water is contained in an enclosed system ("cells") and the remainder ("interspaces") is able to exchange lactate ions with the surrounding saline solution. This conclusion appears, however, to apply only to isolated muscles. For these observations also suggest that although the lactate content of muscles of fresh frogs varies considerably from frog to frog, there appears to be, in the intact animal, a correspondence between the lactate concentration of these two portions (Fig. 2 .). The two values rise and fall together. Since the form of the relationship may depend on a number of factors, no stress is laid here on the particular relationship found in these experiments. But this correspondence seems to make it necessary to suppose, that in the intact animal, in certain circumstances, an exchange of lactate across the membranes between the"cells" and "interspaces" is possible. Such a conclusion seems also to be warranted by the undoubted fact that during severe exercise the lactate content of blood steadily rises due to the passage of lactic acid from the muscles into the blood.

At the same time there is considerable evidence that if the lactic acid content of the blood is high, muscles are able to absorb it continuously from blood (Barr and Himwich, 1923, Himwich, Koskoff and Nahum, 1923). Indeed, Evans et al (1933) have suggested that normally lactic acid continuously passes from the blood into the muscles. But in these cases it may be argued that lactic acid is oxidised on the surface membranes of the "cells" as it is absorbed and hence these facts do not necessarily imply a diffusion of lactic acid into the "cells".

It has been suggested frequently in the past that a change of permeability in the membranes may occur when the muscle fibres contract. Thus Embden and Adler (1922) suggested that the membranes become more permeable to phosphate when the muscle is stimulated. It might have been presumed, therefore, that activity, or perhaps merely a state of tone, permits some equilibration between the two fractions.

Some experiments designed to test this hypothesis did not, however, give results supporting it. In these experiments the diffusion was performed as described before in the case of cooled frog muscles, but at 7° C. At the end of three hours' immersion a single induction shock was given to each muscle by introducing two pin electrodes into the saline. These stimuli were repeated at intervals of 15 minutes, 6 - 8 times. In one experiment short tetani lasting about $\frac{1}{2}$ second were produced at intervals of one hour.[1] The proportion of muscle

1. It is reasonable to assume that even during the short period of the excited state in the muscle in these experiments, lactate equilibrium between "cells" and "interspaces" should be possible if the membranes had become permeable, as "it is justifiable to assume that osmotic equilibrium of substances to which the membrane is permeable takes place practically almost instantaneously" (Bayliss, 1924), owing to very large specfic surface and the very small distances involved.

water concerned in the diffusion of lactate was calculated from the distribution of lactate finally reached, and found to be of the same order as obtained previously in the case of unstimulated muscles.[1)] It appears, then, that at least under the conditions of these experiments, an excited state in the muscle does not permit an exchange of lactate between "cells" and "interspaces". This question is discussed in greater detail at a later stage (p.75)

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1. For the data of these experiments see Appendix E. p. 99.

Relative concentration of lactate in frog blood plasma and muscle.

In view of the relative difference of concentrations between "cells" and "interspaces" of muscle, it was thought desirable to estimate the lactate content of blood plasma and muscle water under identical conditions. Following procedure was adopted. Iliac arteries of a decerebrated frog were exposed by removing the urostyle, ligatured, and then one of the arteries opened proximal to ligature and blood collected in a tube, centrifuged, and plasma separated and weighed. At the same time one gastrocnemius was removed and weighed. The lactate content of the plasma and muscle was determined. Cooled, fresh or fatigued muscles were used. Fatigue was induced by causing isometric contractions of the muscles with single induction shocks, one electrode being inserted under the skin of the snout and the other through the two feet. The stimulii were continued for 2 - 3 minutes after all visible contraction of muscles had ceased. The results of these experiments are given in Table 3. Column (1) and (2) give lactate concentration in muscle and plasma respectively in mgms per 100 gms. It was not possible to keep the frogs completely at rest during the operation of removal of blood and muscle, the lactate figures of cooled frogs are therefore somewhat higher than normal.

The figures in column (3) and (4) give lactate concentration in, 'muscle water' and 'plasma water' on the basis that muscle contains 80% and plasma 90%

TABLE 3. Relative concentration of lactate in blood and voluntary muscles of frog.
(Mg. of lactic acid per 100 g.)

	1 Concentration in muscle	2 Concentration in plasma	3 Concentration in muscle water	4 Concentration in plasma water	5 Concentration in muscle "interspaces". (calculated)	6 Concentration in muscle "cells". (calculated)
1	16	32	19	35	33	10
2	20	42	25	38	43	15
3	21	42	25	38	43	15
4	22	44	28	48	47	20
5	30	57	38	63	60	28
6	178	210	222	231	-	-
7	145	182	181	200	-	-

Experiments 1 -3 were performed on frogs cooled to 2°C overnight, 4 - 5 on fresh frogs and 6 - 7 on fatigued frogs.

For calculation of the values in columns 5 and 6, see text.

of water (Hill and Kupalov 1930). It will be seen that not only the lactate values of plasma exceed those of muscle, but those of the plasma water also exceed the muscle water. And this is true whether the frog is previously cooled, fresh or fatigued. Columns (5) and (6) give the concentration of lactate in the "interspaces" and "cells" respectively, arrived at by splitting up the total concentration in muscles into these two fractions with the help of Fig. 2. A comparison of figures of columns (4) and (5) shows that lactate concentration in plasma water is of the same order as that of the "interspaces".

From this standpoint there would appear to be a free diffusion of lactic acid between the blood and the muscle "interspaces", the relatively lower concentration of muscle being determined by the normally lower concentration in the "cells". In this connection it is interesting to note that Wittgenstein and Gaedertz (1927) have shown that so far as lactic acid is concerned, the cerebro-spinal fluid behaves as a dialysate of plasma, and the same is true of the aqueous humour of the aphakic eye (Fischer 1930). But it must be admitted that the evidence presented here is merely suggestive and is by no means conclusive to show that the passage of lactic acid through the blood vessels is one of free diffusion.

SUMMARY.

1. Observations on the diffusion of d-lactate in and out of resting frog muscle during immersion in a Ringer's solution containing sodium d-lactate show that only about one-third of the muscle water appears to be involved in the diffusion process, the rest appearing to be shut off by membranes impermeable to lactate. These two portions of muscle have been termed "interspaces" and "cells" respectively.

2. In the fatigued muscle, the "interspaces" tend to disappear presumably due to a swelling of the "cells" on account of increased osmotic pressure within them.

3. In heat rigor, nearly the whole of the muscle water becomes available for the diffusion of lactate.

4. An excited state of the isolated muscle does not appear to render the membranes bounding the "cells" permeable to lactate.

5. The normal lactic acid concentration in the "cells" of isolated frog muscle is found to be lower than that in the "interspaces", whether the frog is fresh or has been previously cooled or fatigued. Besides, the muscles appear to maintain a relationship between their lactate concentration in these two fractions, the two values rise and fall together.

6. This relationship in the concentrations in the "cells" and "interspaces" suggests that, although diffusion experiments with relaxed isolated muscles indicate complete separation of "cells" from "interspaces" in respect of lactate, the intact

animal possesses some mechanism by which a relationship between the concentrations in these two portions is maintained.

7. The lactate concentration of muscle is found to be higher than that of blood plasma, but there appears to be a close approximation in the lactate contents of "interspace" water and plasma water.

Diffusion of Iodide into and out of Voluntary muscles of frog.

Methods.

To determine the fraction of the muscle water into which iodide can diffuse, either an isolated muscle (Gastrocnemius) of R. esc (Hungarian) or a thigh preparation, skinned, and separated at its upper end and at knee joint, was employed. In either case the preparation was weighed and immersed in a known weight of modified Ringer's solution in which some of the sodium chloride had been replaced by an equivalent weight of sodium iodide. The time taken to reach diffusion equilibrium between the Ringer and the muscle with respect to iodide was previously determined and found to be about 5 hours in the case of isolated muscles.[1] Diffusion was thereupon allowed to proceed for at least 6 hours in the case of isolated muscles, and for 16 hours for thigh preparations. The muscle was then removed, and the saline estimated for its iodide content.[2] In some cases the iodide content of the muscle was also determined. Some experiments were performed with muscles in heat rigor. Rigor was induced by keeping the preparation at 45°C. for 5 hours. It was then given a preliminary soaking in normal Ringer for a few hours. The preparation was then weighed and the diffusion experiment carried out as in the case of fresh muscles.

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1. Experimental details are given in Appendix G . p.102
2. The method of determination of iodide is described in Appendix F . p 100.

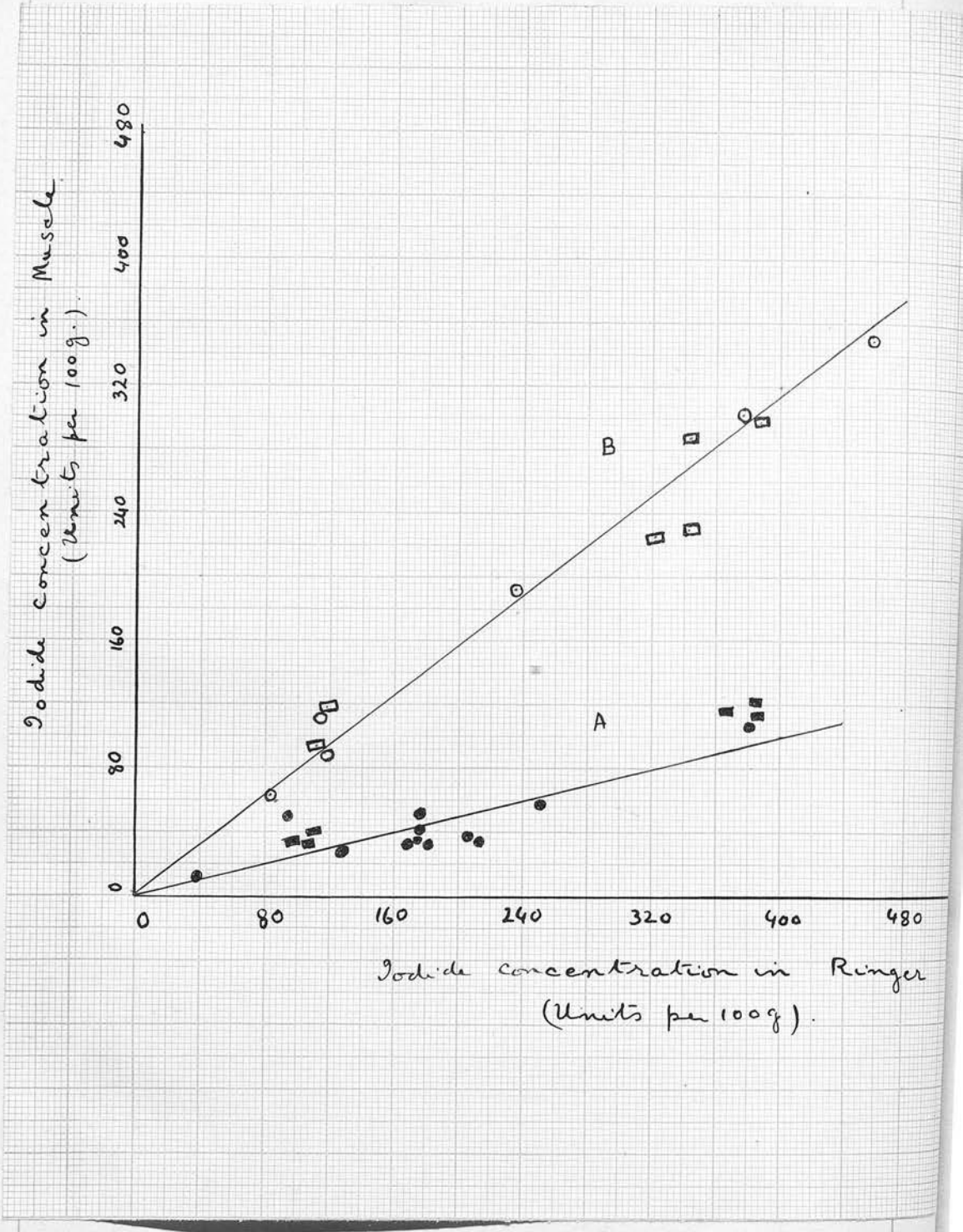

FIG. 3. Relative concentration of iodide in Ringer's solution and frog muscle after the two have reached diffusion equilibrium with regard to iodide.
A - Fresh muscles, ● isolated muscles
■ thigh preparations.
B - Muscles in heat rigor, ○ isolated muscles
□ thigh preparations.
Iodide concentrations given in arbitrary units (1 unit = 1c.c. of N/500 sodium thiosulphate solution required for titration of iodine).

It was not possible to perform experiments on fatigued muscles on similar lines on account of the rather long period required for diffusion equilibrium to be attained, as fatigued muscles tend to pass into rigor if the diffusion experiment is prolonged for more than 4 hours (Hill, 1930).

Results:-[1)]

The results of the diffusion experiments are given in Fig. 3., in which the final iodide concentrations in the Ringer's solution are plotted against the final overall concentration in muscles. The lines passing through the points both for fresh muscles and for muscles in heatrigor prove to be straight, eliminating the possibility of adsorption playing an important part in the diffusion process. Further, the slope of the line A, for fresh muscles indicates that the final concentration of iodide in muscle is only about 24 p.c. of that in the Ringer. Since the total water content of a muscle is about 80 p.c. of its weight, it follows that if the diffusion is a simple osmotic one, only about one third of the total muscle water takes part in the diffusion process. The fraction of muscle water (α) taking part in the diffusion process seems to be a little

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1. For the data of these experiments, and the values of α calculated for each muscle by an algebric operation as in the case of lactate, see appendix H, p. 103-106.

higher in the case of thigh preparations than in isolated muscles but the difference is not sufficiently marked to be decisive.

The line B, for muscles in heat rigor shows that the final concentration in muscle is about 80 p.c. of that in the Ringer, which means that the iodide can diffuse through nearly the whole of the muscle water. It appears that as in the case of lactate the barriers which shut off about two thirds of the muscle water in the case of fresh muscles break down when it passes into rigor.

Coefficient of diffusion of iodide through muscle.

Diffusion of iodide out of the muscle was studied by determining the rate of diffusion of iodide from the fresh and dead muscles. For this purpose hind limb preparations of frogs (either fresh or after being sent into heat rigor) were immersed in a Ringer's solution, in which some of the sodium chloride had been replaced by an equivalent weight of sodium iodide, for 18 hours. At the end of this period an appropriate amount of this Ringer was used to determine the concentration of iodide in it (mg. per c.c.). The thigh preparation was then removed from the saline and immersed into three successive portions of well stirred normal Ringer solution for definite periods. The amounts of iodide diffusing out of the muscles into each of these portions of Ringer's solution was estimated.

The surface area of the preparation was then determined by covering it with strips of paper and noting the total area of paper required to cover it.

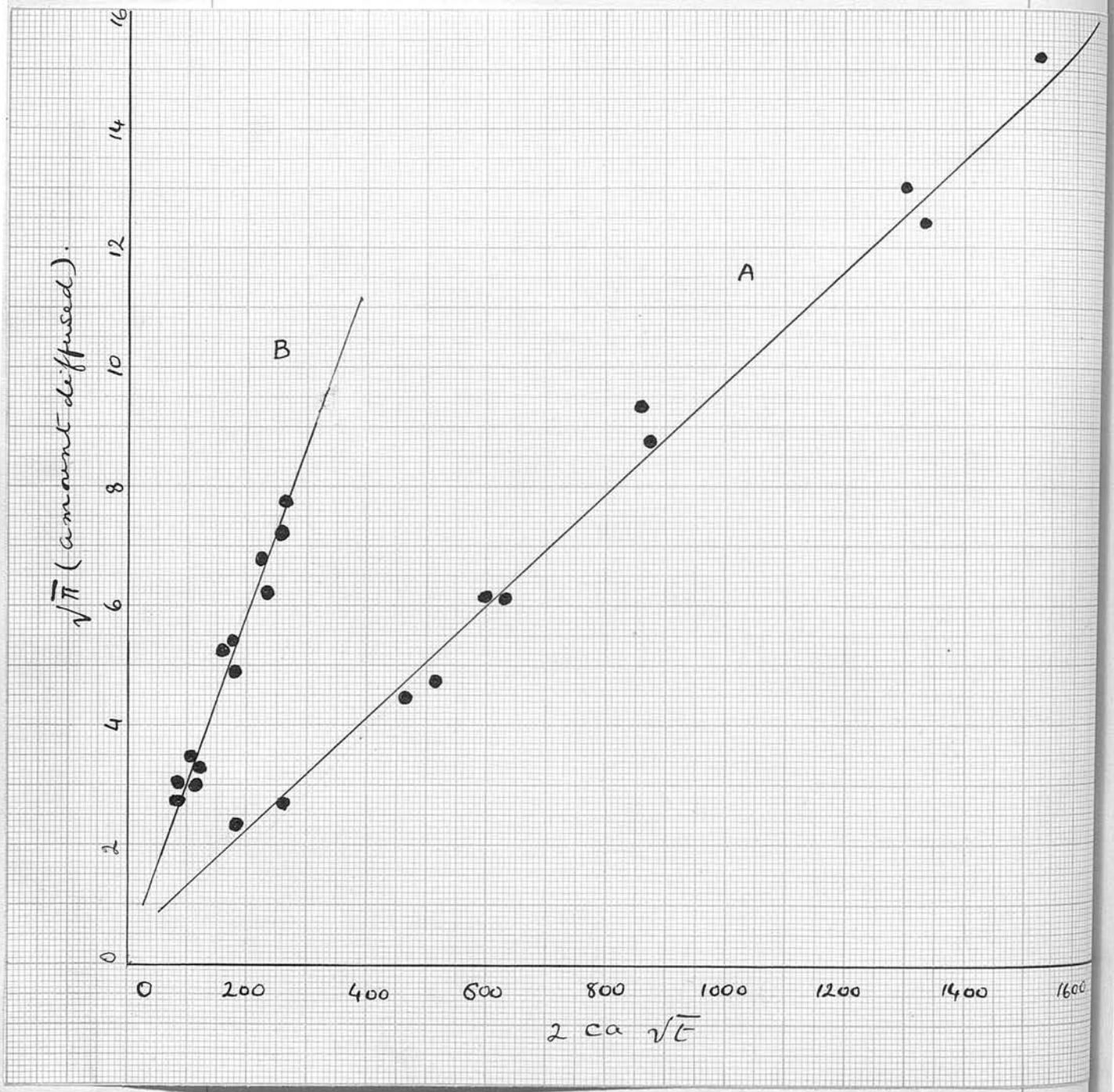

FIG. 4. Coefficient of diffusion of iodide through frog muscles.
A - Fresh muscles.
B - Muscles in heat rigor.

The slope of the lines gives the value of $\sqrt{k}$.

The diffusion of iodide away from hind limb preparations into well stirred Ringer solution was found to follow the square root law applying to semi-infinite solids, agreeing in this respect with lactate (Eggleton, Eggleton and Hill 1928), chloride, sulphate (Conway and Kane 1934) etc. The law can be stated as

$$\text{Amount diffused per Sq. cm. of surface} = 2c\sqrt{\frac{kt}{\pi}} \qquad (1)$$

where c is the initial concentration in the semi-infinite solid, t the time of diffusion and k the coefficient of diffusion to hind limb preparations. It was considered best to take for c, the concentration in a Ringer's solution with which the limbs had been previously equilibrated.

Re-writing equation (1) as

$$\sqrt{k} = \frac{\sqrt{\pi}\ (\text{amount diffused})}{2\,c\,a\,\sqrt{t}} \qquad (2)$$

where a is the measured surface of the preparation, it will be seen that if the amount diffused be plotted against time allowed, the result should be a straight line having slope k. It will be seen from Fig. 4 that the living and dead muscles give results which lead to straight lines, the slopes of which give values of k of 1.2×10^{-4} and 8.5×10^{-4} respectively. Therefore k, the apparent diffusion constant, is 1.2×10^{-4} for living, and 8.5×10^{-4} for dead muscles.[1]

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1. Full data of these experiments as well as the values of k determine algebrically for each experiment are given in appendix. I, p. 107.

"interspaces" of living muscles is 94 p.c. as fast as through free solution.

SUMMARY.

1. Observations on the diffusion of iodide into the muscle whem immersed in a Ringer's solution containing sodium iodide show that in the case of fresh muscle only about one third of its muscle water seems to be involved in the diffusion process, the rest appearing to be shut off by membranes impermeable to iodide. In muscle in heat rigor, however, iodide can diffuse through nearly the whole of its muscle water.

2. The diffusion constants of iodide through (a) fresh muscle of frog previously soaked in Ringer solution containing sodium iodide and (b) muscle in heat rigor similarly treated, are determined and found to be 1.2×10^{-4} and 8.5×10^{-4} respectively. These values indicate that the surface through which diffusion occurs in the case of muscle in rigor is rather less than three times the surface through which iodide can diffuse in fresh muscle. These results support the direct estimations mentioned above.

3. Diffusion constant of iodide through an agar jelly was found to be about 9×10^{-4}.

Diffusion of bicarbonate into and out of voluntary muscles of frog.

Following is an account of some preliminary experiments with the object of the study of the diffusion of bicarbonate into and out of frog muscle. It was originally proposed to study the equilibrium concentrations of bicarbonate in muscle and Ringer's solution containing varying amounts of bicarbonate, and to observe the effect of varying the CO_2 pressure to which the muscle-Ringer system was exposed over a fairly wide range. This work is as yet in a preliminary stage, but the experiments that have been performed throw some light on the condition of bicarbonate in the muscle, and may therefore be described here.

Methods.

The general experimental procedure was that adopted for the study of the diffusion of lactate. Only the gastrocnemii of R. esculenta (Dutch) were used, one of the twin muscles being immersed in a 'high' and the other in a 'low' birarbonate Ringer. Ringer solutions of the required bicarbonate concentrations were prepared by replacing some of the sodium chloride of the Ringer by an equivalent weight of sodium bicarbonate. Special stoppered tubes were used which could be connected in series, so as to allow a gas (CO_2 - O_2 mixture) from one source to

pass through all of them. The arrangement will be clear from Fig. 5. Four such tubes were used in

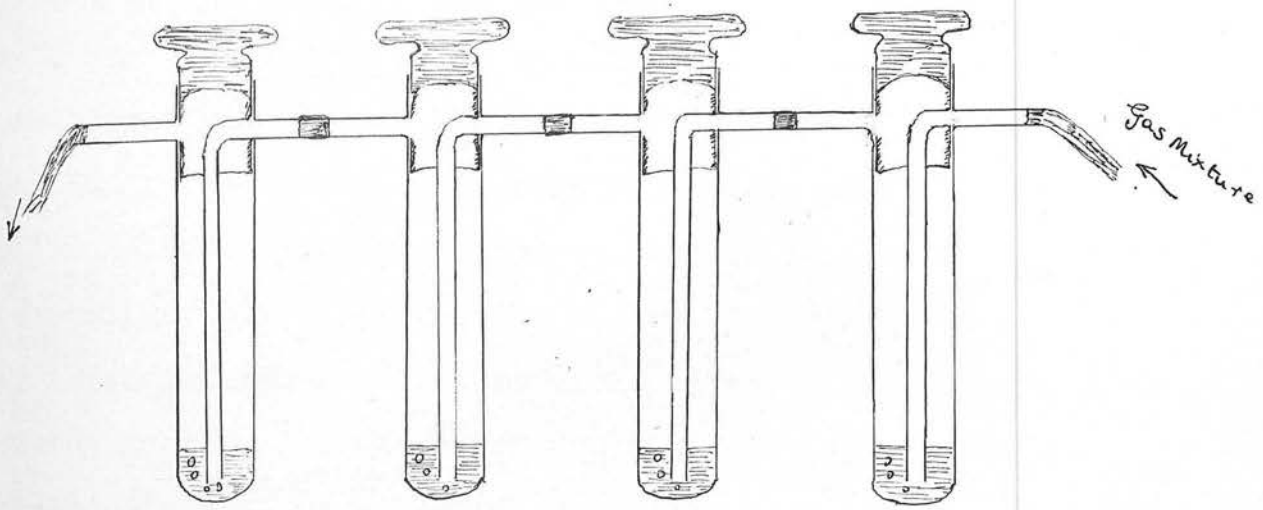

Fig. 5.

each experiment to contain (i, ii) 'high and 'low bicarbonate' Ringer solutions and (iii, iv) the same solutions with muscles immersed in them.

Carbon dioxide — oxygen mixtures were prepared in a bottle above a strong solution of calcium chloride (Sp. gr. 1.40),[1] by exhausting the bottle and then filling it up with the two gases in the ratio required. Calcium chloride solution from a second bottle in communication with the first one was used to force the gas through the tubes containing the

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1. Calcium chloride solution of specific gravity 1.4 was used on account of the low solubility of CO_2 in it — the coefficient being 0.08 as compared to 0.90 for water at room temperature (Peters and Van Slyke, 1931).

gas through the tubes containing the muscle-Ringer systems described above.

The total CO_2 content of the Ringer solution and of muscle was estimated in the first three experiments, with Van Slyke's Volumetric apparatus fitted with a gas-trap as suggested by Shöhl (1923). In the remaining experiments the estimations were performed with the Van Slyke's portable manometric apparatus (Van Slyke, 1927). Estimation of the Ringer's solution by these methods requires no explanation. In the case of the muscle, the dissolved CO_2 was fixed by immersing the muscle in a weak caustic soda solution, and then the total CO_2 driven out by acidifying and boiling the solution and muscle. The CO_2 thus liberated was trapped in a weak CO_2 free solution of NaOH, and estimated by one of the methods described above.[1]

The time required for diffusion equilibrium to be attained between the Ringer and muscle with respect to bicarbonate was determined by a method similar to that adopted for lactate and iodide and found to be about $1\frac{1}{2}$ to 2 hours. But it has been shown by Lipmann and Meyerhof (1930) that in the presence of CO_2 the phosphagen of the resting frog muscle slowly breaks down into creatin and phosphoric acid, and since these products are more alkaline than their parent substance (Fiske and Subbarow, 1929), the CO_2 binding power of excised muscle increases with time.

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1. For details of this method, see Appendix J . p 109.

Root (1933) has shown that this increase in the acid binding power at any CO_2 tension ceases after 5 hours exposure. For greater uniformity of results, it was decided, therefore, to allow diffusion to proceed for 5 hours in all cases.

Temperature control. In the first three experiments the diffusion was carried out at 0°C., but the estimation of CO_2 was performed at room temperature. In the later experiments both the diffusion and estimation were carried out in a cold room at 7°C.

Results.

The total CO_2 content of Ringer in muscle as determined by the method described above consists of two components — the dissolved CO_2, and the bicarbonate.[1] Since we are here concerned with the diffusion of bicarbonate it is necessary to find what fraction of the total CO_2 is in the form of dissolved CO_2. The amount of CO_2 dissolved in water at any partial pressure of CO_2 can be calculated from the absorption coefficient of CO_2 in water (Landolt and Bornstein tables.). Salts depress the solubility of

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1. Strictly speaking there are at least four forms of carbonic acid in the syatem — anhydrous CO_2 H_2CO_3, $'HCO_3$ and $''CO_3$. According to Buytenduyk et al (1927), over 99 p.c. of dissolved CO_2 is in the anhydrous form, and for all practical purposes anhydrous CO_2 and H_2CO_3 may be regarded as one component. The proportion of $''CO_3$ under the conditions of these experiments is negligible, and therefore one is justified in assuming only two components in the system — $'HCO_3$ (bicarbonate) and dissolved CO_2 (usually represented as H_2CO_3).

CO_2 in water and from the solubility curves of CO_2 in salt solutions by Van Slyke et al (1928) it appears that the solubility of CO_2 in Ringer's solution is about 97 p.c. of that of water. Fenn (1928) in determining the CO_2 dissociation curve of frog muscle assumed the solubility coefficient of CO_2 in muscle to be 80 p.c. of that of water. The partial pressure of CO_2 in the experiments is given by the direct estimation of the percentage of CO_2 in the gas mixture. With these data the amount of CO_2 dissolved in the Ringer's solution and in the muscle can be calculated. By subtracting these values from the total CO_2 contents, the bicarbonate contents of Ringer and muscle can be obtained. The proportion of muscle water, α, involved in the diffusion of bicarbonate can then be determined by the algebraic operation used in the case of lactate (P.9).

It is also possible to determine the pH of the Ringer's solution at the end of the experiment by employing the Henderson Hasselbalch equation,

$$pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}.$$

In these determinations the value of pK' was taken to be 6.10 as found for physiological saline solutions by Van Slyke, Sendroy and Hastings (1928).

The average value of α obtained in this way is found to be 35 p.c., showing that the bicarbonate can diffuse into the "interspaces", but not into the "cells", and that relative sizes of the "cells" and the

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1. Details of these experiments and the calculated value of α for each experiment, and pH values of the Ringer are given in Appendix K. p. 111.

"interspaces" are of the same order as in the case of lactate and iodide. The normal bicarbonate contents of these two fractions of muscle water calculated as in the case of lactate (P.14) further shows that bicarbonate is present both in the "cells" and in the "interspaces", but its concentration in the "interspaces" is higher than in the "cells".

The mechanism of poisoning of muscle with iodoacetic acid, with a note on the diffusion of iodoacetate into muscle.

As a preliminary to experiments for the study of diffusion of iodoacetic acid into and out of frog muscle, the question of the possible conversion of some of the iodoacetic acid into an iodide during the course of the experiment had to be considered, as it has been shown by Dickens (1933), Rapkine (1933) and others that when sodium iodoacetate and glutathione are mixed a reaction occurs resulting in the formation of iodide and a thio-ether -

$$G.SH + I.CH_2.COONa \rightleftharpoons HI + S\begin{matrix}G \\ CH_2.COONa.\end{matrix}$$

In an earlier paper Quastel and Wheatley (1932) had shown that glutathione reacts with iodoacetic acid and reduces its toxicity when injected. Since it has been shown by Lohmann (1932) that glutathione is the co-enzyme of glyoxalase, Quastel (1933) postulated from these and other similar observations that the toxicity of iodoacetic acid might be linked with its ability to combine with the sulphydryl compound glutathione, and thus inhibit the glyoxalase activity. Lohmann (1933), on the other hand, showed that the formation of lactic acid from glycogen in glutathione free muscle extracts is inhibited by iodoacetic acid, and came to the conclusion that the poisoning does not depend upon the interaction between iodoacetic acid and glutathione.

In view of the question of the bearing of the iodoacetic acid-glutathione reaction on the mechanism

of the inhibition of lactic acid formation in muscle, it was decided to compare (in the case of frog muscles immersed in Ringer's solution containing iodoacetate), the time relations of the formation of iodide and the effective poisoning of the muscle by iodoacetate (referred to in the following description as I.A.A.). In each experiment the muscles were exposed to I.A.A. Ringer for a certain period at the end of which some were sent into heat rigor, and their lactate content taken as a measure of the degree of poisoning, the rest were used to determine the iodide formed during the exposure to I.A.A. In this way an attempt was made to determine how far the poisoning action of I.A.A. could be explained by the interaction between the acid and -SH compounds with the production of an iodide.

In the course of this investigation some information has been gained as regards the diffusion of I.A.A. into the living and dead muscles, and therefore this work is included in the thesis.

METHODS.

In all the experiments isolated muscles of R. esculenta (Hungarian) were used. The frogs were kept overnight at 0°C. The muscles of each limb, comprising gastrocnemius, sartorius, semimembranosus, gracilis, and semitendinosus, were carefully dissected, weighed and kept in cooled oxygenated Ringer solution for half an hour. Each set of muscles was then removed from the Ringer, blotted and transferred to Ringer solution containing I.A.A. in N/1000 concentration. The pH of this Ringer had been brought up to 7.2 - 7.4 by the addition of a little sodium bicarbonate. The experiment was performed at 0°C and gentle stirring was maintained by bubbling oxygen through the solution. After the required period of immersion, varying from one to ten hours, the muscles derived from one of the limbs, together with the associated Ringer's fluid, were used for the estimation of iodide.[1)]

At the same time the muscles derived from the other limb were caused to pass into heat rigor by transferring them to a water bath at 45°C for one hour. The lactic acid content of this set of muscles was then estimated by the usual method.

L. The method of estimation of iodide is described in Appendix F . p. 100.

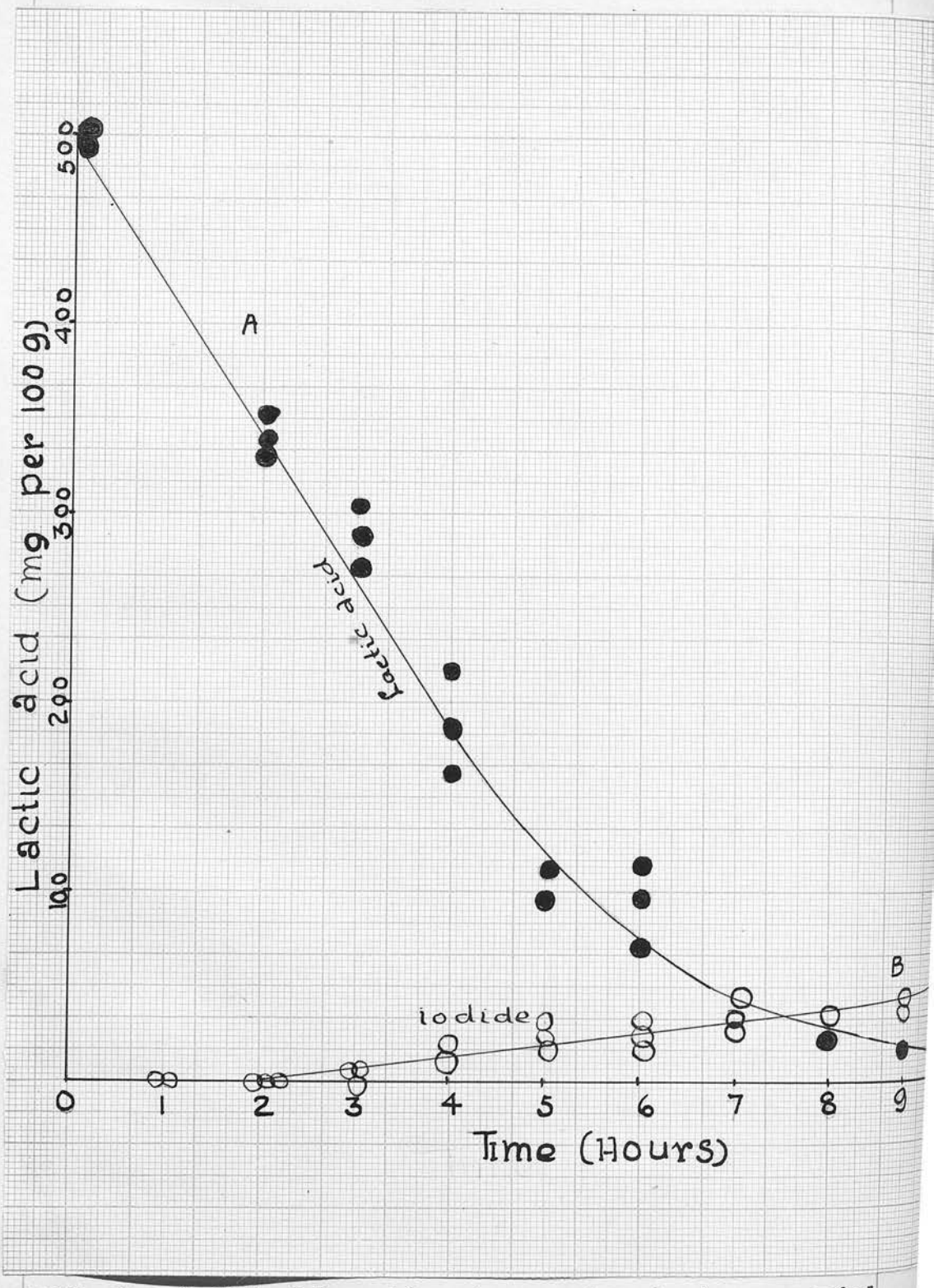

FIG. 6. Iodide liberation in muscles during a period of immersion in Ringer containing iodoacetate, and the degree of poisoning reached in the same period, as measured by the lactic acid produced by similarly treated muscles subsequently sent into heat rigor.
Abscissae - period of immersion in hours.
Ordinates - Curve A, lactic acid produced in heat rigor (mg. per 100g.)
Curve B, iodide liberated in millimols per 100 g. of muscle.
Muscles immersed in oxygenated Ringer containing N/1000 sodium iodoacetate, buffered with $NaHCO_3$, and kept at 0°C.

RESULTS.

The results of these experiments are given in Fig. 6 . Abscissae give the time in hours during which contact between muscles and I.A.A. Ringer was maintained. Ordinates represent the lactic acid formed (in mg per 100 g. of muscle), by those muscles which were subsequently sent into heat rigor, (Curve A), and the amount of iodide formed during the period of immersion in I.A.A. Ringer, (in millimols per 100 g. of muscle), (Curve B). It will be seen that the amount of lactic acid formed in these muscles falls steadily and practically reaches zero in 8 to 9 hours, at the end of which period the lactic acid content of the muscles in heat rigor is equal to the normal resting content (15 - 20 mg.).

The iodide content, on the other hand, is zero till the end of the first two hours (during which period the power to form lactic acid has already fallen to about two-thirds of normal). From the third hour onwards there is a small and variable appearance of iodide, which rises only to an average of 0.008 millimols per 100 g. at the end of 10 hours.

It may be noted here that in the above experiments, by the end of the third hour, the muscles do not appear to be in a good condition. They tend to be rolled up and contracted, possibly some of the fibres having passed into rigor. If the muscles be immersed in Ringer containing half the strength of I.A.A. used in the above experiments (i.e. N/2000 solution), they remain in a good condition for 5 to 6 hours and

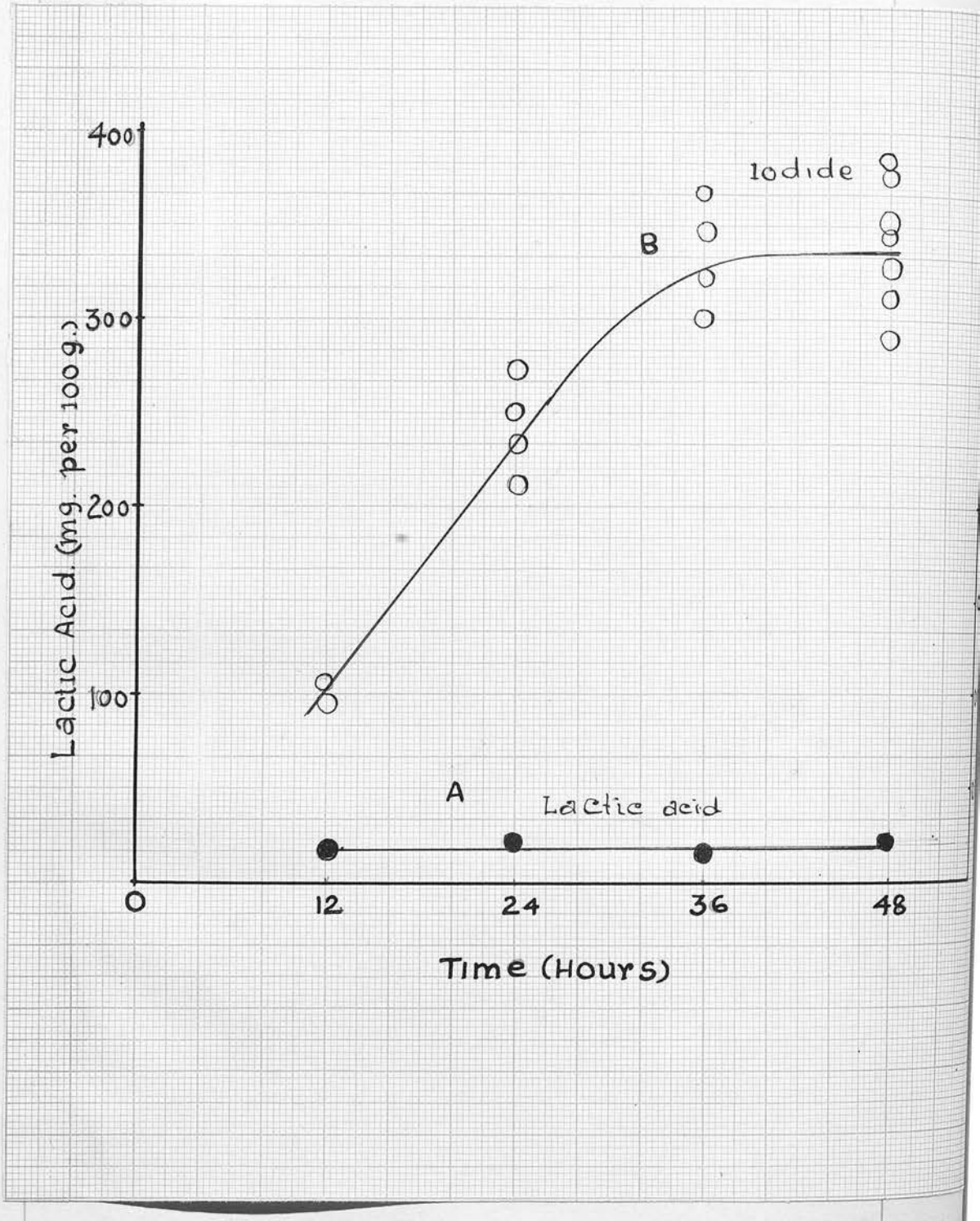

FIG. 7. Experiments of the same type as those of Fig. 1, carried out at room temperature instead of 0°C. See legend of Fig. 1. for details.

generally no iodide appears during this period, although there is a partial loss of glycolytic power.

Some experiments were performed with the same technique as described above except that the muscles were kept at room temperature and the contact with I.A.A. Ringer was maintained for longer periods, to note the maximum amount of iodide that could be formed by the muscles. Under these conditions the muscles pass spontaneously into well marked rigor in about 12 hours. The results are given in Fig. 7 . Judged by the glycolytic activity, the muscles were completely poisoned even in the shortest period (12 hours) used. But the iodide content of the muscle-Ringer system continues to rise till a maximum is reached in about 36 hours, when there is an average of about 0.07 millimols of iodide per 100 g. of muscle. Assuming that the iodide is formed by an interaction between I.A.A. and glutathione, this amount requires the presence of 26 mg. of glutathione per 100 g. of muscle, and this figure is of the same order as that found for the muscles of amphibia by direct estimation, for example by Kamiya (1928).

But the fact that the iodide does not begin to appear in the previous set of experiments till the third hour of immersion when the power of lactic acid production of the muscle is already considerably impaired; and the small amount produced even at the end of 10 hours when the muscle is completely poisoned (Fig. 6 .), makes it appear improbable that the inhibition of the power of lactic acid production depends on

the reaction of iodoacetic acid with glutathione. For taking the amount of iodide produced as an index of the extent of disorganisation of the glutathione system, it is evident that when the muscle has been fully poisoned not more than 10 p.c. of the total glutathione has been destroyed.

It may be argued that even 10 p.c. destruction of glutathione could be sufficient to stop the glycolytic activity of the muscles, if most of the glutathione in the tissue constituted a reserve, but it seems improbable that the muscle should pass into rigor for want of glutathione whilst it still contains a large store of this substance. On the other hand the fact that the iodide usually begins to appear only after the muscle has passed into an unhealthy condition suggests that possibly the appearance of iodide is connected in some way with the death of the fibres, and it seems reasonable to suppose that the living muscle cells are surrounded by membranes impermeable to I.A.A. but the death of the cells allows the acid to pass in and thus come in contact and react with glutathione.[1)]

Reference was made above to the fact that the amount of iodide formed after rigor corresponded approximately to the amount of glutathione known to be

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1. It has been shown by King, Baumgartner and Page (1930), that in blood, glutathione is confined to blood corpuscles, none being present in the plasma. This and the above observations suggest that in muscle also, glutathione is confined inside the fibres.

TABLE 4.

Iodide produced by frog muscles when immersed for 48 hours at room temperature in Ringer's solution containing different amounts and varying concentrations of sodium iodoacetate.

	Concentration of Iodoacetate	Millimols of iodoacetate per 100 g. of muscle.	Iodide Produced Millimols per 100 g. of muscle
1.	N/1000	1,0	0.061
2.	N/1000	1.5	0.058
3.	N/1000	2.0	0.065
4.	N/500	2.0	0.074
5.	N/500	3.0	0.065
6.	N/500	4.0	0.072

present in such muscles. This amount was always a small proportion of the total quantity of I.A.A. supplied, so that it was unlikely that the iodide formation was limited by the supply of I.A.A. To make sure of this point, a few experiments were performed, varying the volume and strength of the iodoacetate Ringer in which the muscles were immersed, and estimating the iodide produced at the end of 48 hours. The results are given in Table 4. It will be seen that given an excess of iodoacetic acid, the concentration of I.A.A. and the total amount per gram of muscle has no influence on the total iodide produced.

The rate and extent of I.A.A. poisoning of frog muscle when kept at 40°C for varying periods was also studied. Some glycolysis always occurred in the first half hour of immersion, probably because I.A.A. takes some time to diffuse into the muscle. Accordingly the general plan was to immerse two corresponding sets of muscles of a frog in (a) normal Ringer and (b) I.A.A. Ringer, both previously warmed to 40°C and kept at that temperature for a definite period. The effect of I.A.A. is then given by the difference in the lactic acid content of the muscle immersed in normal Ringer, and that of the muscle immersed in I.A.A. Ringer. Fig. 8, Curve A gives average <u>difference</u> of lactic acid content between the muscles in the normal and I.A.A. Ringer respectively in mg. per 100 g. of muscle. The difference in lactic acid content between poisoned muscle and

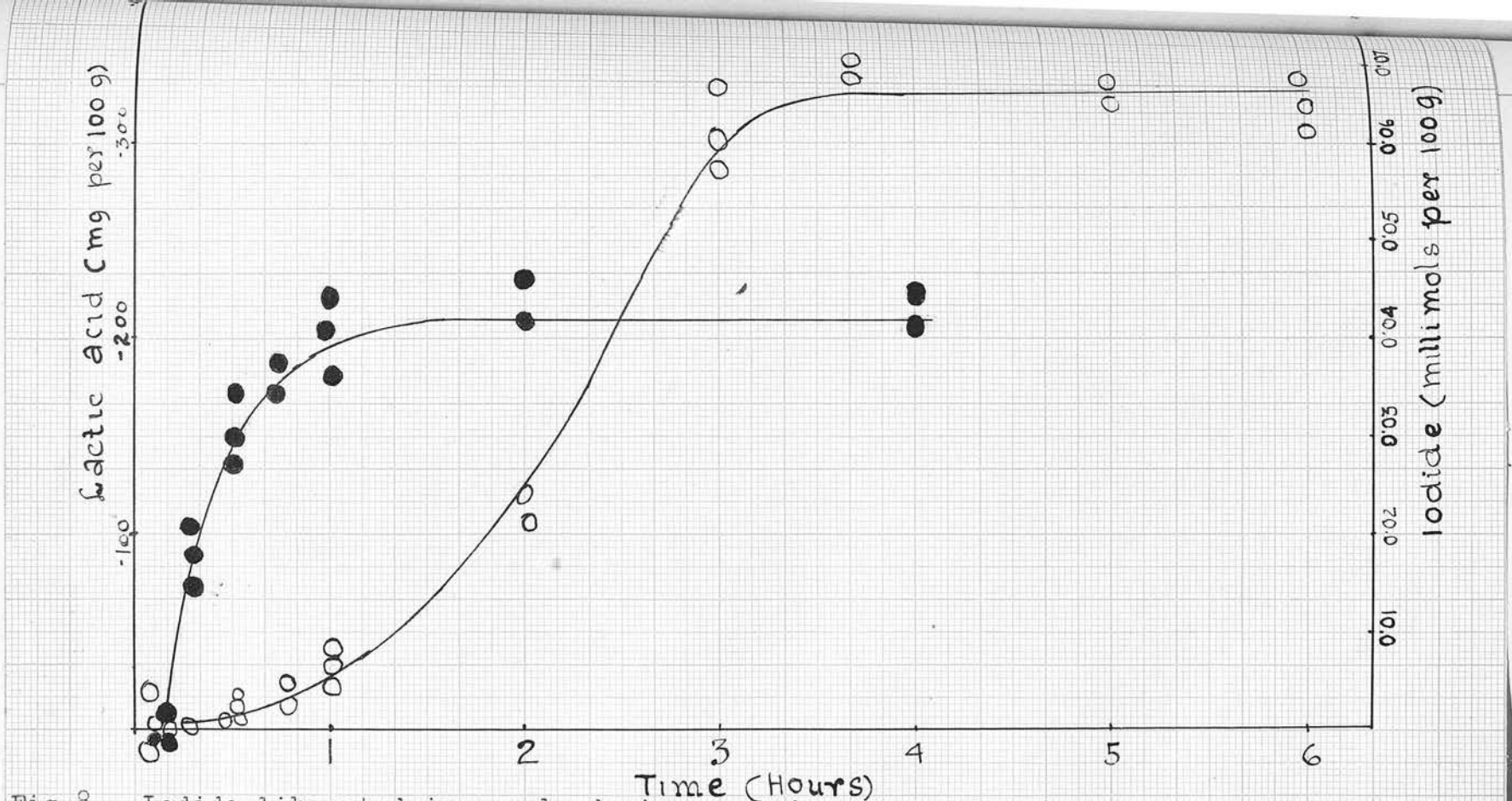

Fig 8. Iodide liberated in muscle during a period of immersion in Ringer containing iodoacetate, and the degree of poisoning reached in the same period.
Abscissae - Period of immersion in hours.
Ordinates - Curve A, - Depression of glycolysis as indicated by the difference in lactic acid content between poisoned and unpoisoned muscles kept at the same temperature.
Curve B, - Iodide liberated in millimols per 100 g. of muscle.

Muscles immersed in Ringer containing N/1000 sodium iodoacetate and kept at 40° C.

control becomes measurable even in ten minutes and reaches its maximum in about 45 minutes. It may be remarked in passing that the poisoned muscles ceased producing lactic acid in about 35 - 40 minutes and the maximum then attained was about two-thirds of the normal maximum.

Curve B, Fig.8 , gives the iodide content of muscles (in millimols per 100 g. of muscle) immersed in iodoacetate Ringer at 40°C for various periods. It will be seen that the iodide begins to appear only after about 20 minutes' immersion and rises slowly till a maximum is reached in 3 to 4 hours. In this case again there appears to be no relation between the formation of iodide (presumably indicating disorganisation of glutathione system) and the inhibition of lactic acid forming power of muscle, for though there is a pronounced poisoning effect in one hour, the reaction responsible for iodide production has only proceeded to 10 p.c. of its total in this time.

SUMMARY.

1. Isolated muscles of Rana esculenta were immersed in Ringer's solution containing iodoacetate (N/1000), and the effect of the drug was followed in two ways: (1) the suppression of glycolytic activity and (2) the liberation of iodide (presumed to result from an interaction between iodoacetic acid and glutathione of muscle).

2. At 0° C. the glycolytic activity of muscle is reduced to two-thirds of normal in two hours, and is completely suppressed in 8 to 9 hours. But no iodide is liberated during the first two hours of immersion, and even at the end of 8 to 9 hours the amount produced is less than 10 p.c. of the maximum that the muscle is capable of liberating.

3. This maximum iodide liberation requires about 36 hours contact between iodoacetate and muscle at room temperature, and amounts to about 0.07 millimols per 100 g. of muscle, irrespective of the amount and concentration of iodoacetate in the system, provided there is an excess. This quantity is equivalent to 25 mg. of reduced glutathione per 100 g. of muscle.

4. At 40° C. the inhibition of lactic acid production becomes apparent in about 10 minutes, and maximum poisoning effect is attained in 30 to 40 minutes. Liberation of iodide, on the other hand begins in about 20 minutes and the maximum production takes as much as 3 to 4 hours.

5. These results do not support the view that the suppression of glycolytic activity of muscle

depends upon an interaction between iodoacetic acid and glutathione, although they do not rule out the possibility of the formation of an additional compound.

6. Reasons are given to suggest that iodoacetate is unable to diffuse into the living muscle fibres, but can do so when these have passed into rigor.

DISCUSSION

I

Histological chemistry of the voluntary muscle of frog

An account of the study of the diffusion of certain dissolved substances into and out of voluntary muscle of frog when immersed in a Ringer's solution, is given in the foregoing pages. In the case of iodoacetate attention was mainly diverted to a study of the reaction of iodoacetate with glutathione to form an iodide, a feature that complicates the study of its diffusion into the muscle. But these observations did give an indication that probably iodoacetate is not able to diffuse into the living muscle cells, but can do so when they pass into rigor. The diffusion behaviour of the other ions studied, namely, lactate, iodide and bicarbonate, presents certain common features. So far as the diffusion of these ions is concerned, the total muscle water may be supposed to consist of two fractions, (1) Water in the "interspaces" forming about one third and (2) that of the "cells" comprising the remaining two thirds of it. In the case of each of these ions it was shown that there was a free diffusion between the Ringer and the "interspace" water, but under the conditions of the experiment there was no exchange of these ions between the "interspaces" and the "cells". It was further shown that the lactate and the bicarbonate are normally present in both these fractions of the

muscle water, but in both cases the concentration of the "interspaces" seems to be higher than that of the "cells".

A number of investigators have studied the diffusion or "permeability" of various substances into the frog muscle. It is now necessary to examine some of these results on the lines of the present investigation in order to form some conception of the histological chemistry of the muscle from the data available.

1. Substances for which there is evidence that they can diffuse into and out of the whole of the muscle water.

(a) Carbon dioxide.

CO_2 can penetrate certain living tissues with great ease (Jacobs, 1920). The coefficient of diffusion of CO_2 in muscle has been determined by Krogh, (1919), Fenn (1928) and Wright (1934). The diffusion constant of Krogh is somewhat higher, and of Fenn somewhat lower, than that of Wright. The last investigator, after careful observations came to the conclusion that CO_2 diffuses through the muscles approximately 65 p.c. as rapidly as through water. As the coefficient of diffusion is proportional to the square of the surface through which diffusion can take place (see p. 32), if it be supposed that CO_2 can diffuse through the whole muscle water without experiencing any hinderance due to membranes, but not through the non aqeous fraction, then, since the muscle contains 80 p.c. of water, the

surface through which diffusion will take place may be considered to form 80 p.c. of the total surface area of the muscle. The diffusion constant of CO_2 through the muscle would then be $(80)^2/(100)^2$ or 64 p.c. of that of water - a figure pratically identical with that obtained by Wright.

This means that the rate of diffusion of CO_2 through muscle water is practically identical with free diffusion through water, and suggests that CO_2 experiences no hinderance in diffusing through the whole of muscle water.

(b) Oxygen.

Harvey (1923) has demonstrated that oxygen can freely diffuse through certain animal and vegetable cells. Krogh (1919) has determined the diffusion constant of oxygen in tissues. He finds that the diffusion constant (amount of gas diffusing through 1 square cm. of surface and 1 mm of thickness in one minute at pressure difference of 1 atmosphere) of oxygen through muscle is 0.14. Expressed in the same units, diffusion constant through water is 0.34 (Hüfner 1897).

If oxygen were able to diffuse through the whole of muscle water, as through a free solution, then, as explained in the case of CO_2, its diffusion constant through muscle would be 64 p.c. of that through water. In other words with a diffusion constant of 0.34 through water, that through muscle under these conditions, should be about 0.21. A diffusion constant of 0.14 would be

obtained if 85 p.c. of muscle water were available for diffusion. This does not necessarily mean the inability of oxygen to diffuse through 15 p.c. of muscle water, but may simply mean that the cell membranes offer additional resistance to the passage of oxygen. As a matter of fact the magnitude of the diffusion constant suggests that oxygen can diffuse through the whole of muscle water.

(c) Urea.

Overton (1902) showed that urea added to an isomotic solution does not cause any permanent change in the weight of the muscle immersed in the saline, which suggests that urea can diffuse into the "cells", so that their osmotic pressure becomes equal to the saline containing urea. Eggleton (1930) studied the diffusion of urea into and out of a muscle immersed in a Ringer's solution and found that on equilibrium being reached, the concentration of urea inside the muscle was about 80 p.c. of that of the saline. As muscle contains 80 p.c. of water this means that urea can diffuse uniformly through the whole of the muscle water.

(d) Histidine.

Diffusion of histidine into and out of muscle was studied by Eggleton and Eggleton (1933). They found that on equilibrium being reached, the concentration of this substance in the muscle was about 80 p.c. of that of the surrounding saline. This implies that histidine can diffuse into the whole of the muscle water.

(e) Potassium.

Mond and Amson (1928) found that muscle fibres were permeable to K ions. Ernst and Takaks (1931) showed that muscles perfused with cane sugar solution lose very little of their potassium content at a time when they have lost most of their Na and Cl. Their observations support the view generally held that most of the potassium is contained within the fibres, where it seems to be 30 - 40 times the concentration of the tissue spaces (Mond and Nettar, 1930, 1932).

Fenn and Cobb (1934) studied the diffusion of potassium in and out of muscle when immersed in Ringer's solution containing varying concentrations of potassium. They found that in the living muscle at pH 7.2, a concentration of 19 mg. per 100 g. of K outside was in equilibrium with the K inside (about 325 mg. p.c.). At lower concentrations of K outside, muscle loses K, at higher concentrations it gains: a new equilibrium being established in either case, and the loss or gain was of such magnitude as to suggest that K must diffuse into or out of "cells". Thus at a concentration of 10 mg. p.c. outside the concentration of K in the muscle falls by 40 mg. p.c. and at a concentration of 30 mg. p.c. outside it rises by 36 mg. p.c.; but even when the concentration of K outside is 0, the muscle still retains about 230 mg. p.c. of it, suggesting that a large fraction of K inside is bound and unable to diffuse out. But one interesting fact that is seen from their observations is that when the K concentration outside rises above

50 mg. p.c., the diffusion thereafter into the muscle is greatly diminshed and amounts to diffusion into the "interspaces" only, if the latter be supposed to form about 25 p.c. of muscle.

These authors suggest that at such high concentrations outside, the membranes become impermeable to K. These observations show that although K can diffuse into and out of "cells", the relative size of "interspaces" and "cells" is of the same order as observed in the case of the other ions considered above.

(f) Other substances which can diffuse into the "cells".

Effect of increasing the osmotic pressure of saline on the weight of muscle suspended in it has been made use of by Overton (1902) to test whether the substance added can diffuse into the muscle fibres or not. For if the substance added cannot diffuse into (a part or the whole of) the muscle fibres, the latter must give up water and shrink in order to increase their osmotic pressure by loss of water.

If on the other hand, the substance added can diffuse through the whole of muscle water, the weight of muscle remains unchanged. In the latter case α must be 100. It is not possible, of course, from these experiments to determine α for those substances which do not diffuse into the whole of muscle water. Overton has tried a large number of substances. Among those that seem to diffuse into the whole of muscle may be mentioned methyl and ethyl alcohol, divalent alcohols (e.g. ethylene glycol), glycerine; amides e.g. urea.

2. Substances that can diffuse into and out of a fraction of the muscle water only.

This group may be subdivided as follows:

(A) Substances that are not present inside the "cells" in the living muscle - sodium, chloride, sulphate and iodide. Glucose may also be provisionally included in this group.

(B) Substances that are normally confined to the "cells" and unable to diffuse out.

Carnosine belongs to this group. A large fraction of potassium probably belongs to this group.

(C) Substances that are normally present in the "cells" as well as in the "interspaces", but but under experimental conditions there appears to be no exchange between these two fractions of muscle water. This group comprises phosphate, lactate, creatine, bicarbonate and possibly glucose.

It may be mentioned here that a number of other substances e.g. bromide, thiocyanate, calcium, and lithium appear to be unable to diffuse into the whole of muscle substance (Mond and Amson, 1928), but as in the case of the observations of overton referred to above, it is not possible to calculate the value of α from these observations.

A. Substances that are not present inside the "cells" in the living muscle.

1. SODIUM

Urano (1908a, b) and Fahr (1909) observed that

nearly the whole of the Na content of muscle can be removed by washing with saccharose solution.

Mond and Amson (1928) showed by perfusion experiments that muscle fibres are impermeable to sodium.

Mond and Nettar (1932) suggested that there is no sodium inside the muscle fibres, but it is bound up on the surface of muscle. Fenn, Cobb and Marsh (1934) showed that the concentration of sodium in muscles (Sartorii) soaked in Ringer's solution in which some of the sodium chloride had been replaced by dextrose, was regularly about 33 p.c. of the concentration in the saline, and that the experimental points when plotted fall along a diagonal. This would mean that the value of α was about 40 p.c., and that there was no appreciable amount of sodium inside the "cells".

They further contended that there was no adequate reason for assuming as Mond and Nettar did, that sodium was bound up on the surface of the fibres, but give reasons to believe that the distribution of Na in the muscle of intact animal might be different from what obtains in isolated muscle soaked in Ringer. This point will be discussed at a later stage.

2. Chloride.

It has been observed by a number of investigators (Urano, 1908; Thompson, 1928), that muscles washed with cane sugar solution lose nearly the whole of their chloride content. Fenn, Cobb and Marsh (1934) showed that the concentration of chloride in the

muscles after 5 hours soaking in the Ringer's solution, (the chloride content of which was varied by replacing some of the sodium chloride by an equivalent amount of sodium nitrate), was regularly about 31 p.c. of the concentration in the Ringer.

Supposing the equilibrium to be a simple osmotic one, this means that about 38 p.c. of the muscle water is involved in the diffusion process — α is 38 in the case of the sartorius of frog. Conway and Kane (1934) also observed about the same relative concentration of chloride in Ringer and muscle, though these authors give a different interpretation to their results. Eggleton and Eggleton (1935) by using the larger muscles of frog observed that only about 30 p.c. of the muscle water was involved in the diffusion process.[1] They further showed that the chloride was normally confined to the "interspaces" only, none being present in the "cells"

3. SULPHATE.

Conway and Kane (1934) determined the concentration of inorganic sulphate in muscles in diffusion equilibrium with a definite concentration of sulphate in the surrounding saline, and found it to be about 25 p.c. of the latter. As the muscle contains about 80 p.c of water, this means that the concentration of sulphate in muscle water would be about one third of of that in the saline. It is reasonable to suppose

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1. The reason of the slightly lower value of obtained by these authors as compared to that of Fenn, Cobb and Marsh is discussed at a later stage.(p. 71).

that sulphate, like chloride, can diffuse into the "interspaces" only, and these contain about one third of the total muscle water.

4. IODIDE.

The diffusion of this ion has been discussed already, and need not be repeated here.

5. GLUCOSE.

Overton showed that a muscle immersed in an isomotic saline in which some of the sodium chloride had been replaced by an equivalent weight of glucose did not show any appreciable change in weight even on prolonged soaking. This means that glucose is not able to diffuse into the "cells", for if this were the case, the saline would have behaved like a hypotonic solution towards muscle and the muscle would have increased in weight. Cori, Closs and Cori (1933) showed that the concentration of fermentable sugar in blood plasma of rats was 5 - 10 times higher than in muscle, one explanation of which would be that glucose is not able to diffuse into a large fraction of muscle. M. G. Eggleton (1935) has studied the diffusion of glucose into and out of voluntary muscle of frog when immersed in Ringer's solution containing glucose. It was found that even on prolonged washing a small fraction of copper reducing substance was still present in the muscle, but whether it was glucose or some other product was not certain. Neglecting this fraction, the concentration of glucose in the muscle in diffusion equilibrium with the Ringer was found to be about

25 p.c. of that in the saline, which means that in this case also, only about one third of the muscle water was involved in the diffusion process.

B. Substances normally confined to the "cells".

(1) Carnosine. Eggletons (1923) found that a frog muscle immersed in Ringer's solution lost none of its carnosine. Carnosine added to the Ringer could diffuse into and out of muscle, but at equilibrium the concentration of the extra carnosine in the muscle was only 30 p.c. of the concentration in the Ringer outside, suggesting that only 30 p.c. of the muscle water took part in the diffusion system. This in turn makes it appear likely that the muscle's own carnosine is contained within the "cells" occupying about 70 p.c. of the volume of the muscle.

(2) Potassium. The diffusion of this ion has been discussed in Group I. A large fraction of potassium seems to be unable to diffuse out from the "cells", and this fraction may be regarded to belong to this group.

C. Substances normally present in the "cells" as well as the "interspaces".

(1) Phosphate.

Ernst and Takacs (1931) observed that muscles perfused with saccharose solution lost very little of their PO_4 content, which suggested that the greater portion of this ion was confined within the "cells". This observation apparently conflicted with that of the Stella (1928) who in a study of the exchange of phosphate between the Ringer and hind

limb preparations of frog came to the conclusion that the phosphate was able to diffuse from the Ringer into the whole of the muscle water. M. G. Eggleton (1933) showed that the high values of α obtained by Stella could be explained by the fact that he employed muscle-bone preparations, and that the bone was able to absorb large amounts of phosphate from the surrounding saline. The value of α in isolated muscles was found to be 20 - 30 p.c. A corroboration of this finding is obtained in the work of Semeanoff (1931) who determined the concentration of phosphate in the Ringer that would prevent the loss or gain of phosphate from the Sartorius immersed in it. The value of α can be calculated from her results and gives an average of 39. It appears then, that inorganic phosphate is unable to diffuse into or out of the "cells" and the relative sizes of the "interspaces" and "cells" are of the same order as found for chloride, lactate etc.

(2) Creatine.

Overton (1902) found that a muscle immersed in an isomotic saline in which some of the sodium chloride had been replaced with an equivalent weight of creatine, did not show an appreciable change of weight even on soaking for several hours. This suggests as in the case of glucose that creatine is not able to diffuse into the "cells". Eggleton (1930) determined the concentration of creatine in Ringer solution that is just sufficient to prevent loss or gain of creatine from muscle. From his

data it is possible to calculate the value of α, by the algebraic operation used for the determination of α for lactic acid. The value thus calculated is found to be about 30 p.c.

(3) and (4)

Lactate and Bicarbonate have already been discussed in the preceding pages.

This resumé of the diffusion of various substances through the living voluntary muscle shows that those examined up to this time can be divided into two broad groups: (1) Substances that can diffuse into the whole of muscle water, and (2) those that can diffuse only into the "interspaces" of the muscle, but not into the "cells". It is further shown that in all cases where the relative sizes of "interspaces" and "cells" have been determined, the former appear to contain roughly one third and the latter about two thirds of the total muscle water. Adsorption does not seem to play a significant part in the process in these cases, nor is there any evidence of membrane equilibrium of Donnan type, except in the case of potassium where a membrane equilibrium somewhat different from Donnan equilibrium (Netter, 1928) seems to be concerned in determining the concentration inside and outside the "cells". It is suggestive though by no means rigidly proved, that the membranes preventing the diffusion of various substances between "cells" and "interspaces" are common for all the substances considered here.

In the following table an attempt has been made to give the concentration of various diffusible substances in the "cells" and the "interspaces" by splitting the known values of gross concentration in muscle into these two fractions according to their relative concentrations as found by various workers.

TABLE 5. Normal concentration of various substances in total muscle water, in the "interspaces" and the "cells".

	Gross concentration in muscle water mg. per 100g.	Concentration in the "interspaces". mg. per 100g.	Concentration in the "cells"" mg. per 100g.	Investigator from whose data the Relative concentrations have been calculated.
Chloride	50	180	0	Eggleton and Eggleton (1935)
Sodium	83	249	0	Fenn, Cobb and Marsh (1934)
Potassium	412	19	608	Fenn and Cobb (1934)
Inorganic phosphate	18	12	21	M.G. Eggleton. (1933)
Lactate	20	37	12	Ghaffar
Carnosine	190	0	285	Eggleton and Eggleton (1933)
Carbon dioxide	3.4*	3.4	3.4	Wright (1934)
Bicarbonate	69+	117	44	Ghaffar
Creatin	80	80	80	P. Eggleton (1930)
Sugar	10	30	0?++	M.G. Eggleton (1935)
Urea	20	20	20	P. Eggleton (1930)

* Fenn (1928) + Root (1933) ++ see p.64.

II. Conditions that may cause a change in the value of α.

The statement that the "interspace" water is about one third of the whole muscle water applies only to the average value of resting isolated muscles of frog immersed in isotonic Ringer's solution. It is now proposed to consider the changes in α that may occur under different conditions.

(i) Variations in the value of α in the same muscle of different frogs. The values of α determined for a single muscle e.g. gastrocnemius usually vary within a fairly wide range. Thus in the case of lactate the values of α range between 28 and 48 p.c. Considerable variation may be due to experimental error as the calculations are based on small differences, the number of measurements in any one experiment is large, and in calculation the experimental error gets multiplied. But there is evidence that these differences in the value of α are partly due to actual differences in the gastrocnemii of different frogs. It was observed that in calculating the concentration of lactate in the "cells" and the "interspaces" (P.14) if the value of α were supposed to be the average obtained (35 p.c.), the values obtained for the two twin muscles gave widely different results, while if the actual value of α calculated for that pair was employed, the difference was comparatively small. This suggests that α may vary in the gastrocnemii of different frogs within a fairly wide range.

(ii) Differences in the different muscles of frog. It was observed by Eggleton and Eggleton in studying the diffusion of chloride, that the average values for α in the sartorius and the gracilis minor were persistently higher than that of the larger muscles — gastrocnemius, semi-membraneous and gracilis major. This may be the reason of the rather high value of α (about 40 p.c.) obtained by Fenn, Cobb and Marsh in studying the diffusion of sodium and chloride, as they used the sartorius in their experiments.

(iii) Change in the value of α on immersing the muscles in hypotonic or hypertonic saline. When a muscle is immersed in hypotonic or hypertonic saline, it swells or shrinks proportionately to the molar concentration of the saline used (Beutner, 1912). If the membranes regulating the passage of water into and out of the muscle are formed by interfaces between the "cells" and "interspaces", then it may be expected that α should decrease when the muscle is placed in hypotonic solution, and increase when the "cells" shrink on immersing the muscle in hypertonic solution. This has actually been shown to be the case by Eggleton and Eggleton in experiments on the determination of α for chloride.

(iv) Effect of fatigue. Fatigue of the muscle causes a rise of osmotic pressure inside the muscle, so that an isolated fatigued muscle is in osmotic equilibrium with a Ringer solution containing 1.2 p.c. NaCl instead of 0.71 p.c. NaCl as in the case of

resting muscle (Hill, 1930). Consequently it is found that muscles in the intact animal swell as a result of fatigue (Barcroft and Kato 1915, Bock Cogan and Towers, 1915). A fall in the value of α may therefore be expected in the fatigued muscle. Experimental results are not decisive on this point. M.G. Eggleton observed little change in α in fatigued muscles in the diffusion of phosphate and glucose. The writer, on the other hand, observed a marked decrease in the value of α in fatigued muscles in experiments with lactate. One possible explanation of this difference in results may be the different conditions of experiments. Eggleton fatigued the muscles in the isolated limb and used Ringer containing 1.2 p.c. NaCl for immersion. Under these conditions there may not be a swelling of the "cells" to any marked degree. The writer stimulated the limbs with the circulation intact so that muscle fibres could swell by absorbing water from blood. The muscles were, after dissection, immersed in 0.7 p.c. NaCl Ringer as this saline is found to be isosmotic with muscles fatigued with the circulation intact (Moore, 1916). This mode of treatment may be expected to cause greater swelling of "cells" and a corresponding fall in the value of α.

(v). Muscle in heat rigor. It is well known that death of cells, especially by heat, destroys the semipermeability of their membranes (Osterhout, 1912) This appears to be the case with muscle "cells" also, for it has been shown that phosphate, chloride,

iodide, lactate and carnosine can all diffuse through nearly the whole of the water of the muscle in heat rigor. In other words, α tends to be 100 in this condition.

(vi) The value of α in the living muscle when the immersing fluid is serum or lymph instead of the Ringer. All the experiments discussed above in which the value of α has been determined, concern isolated muscles immersed in Ringer's solution. Eggleton and Eggleton performed some diffusion experiments on the same lines but substituted serum for Ringer. The value of α fell to about 15. A difference of another kind was observed by Fenn, Cobb and Marsh (1934) in the sodium and chloride contents of muscle from intact frog as compared to these values for muscle immersed in Ringer. The average value for Na and Cl in intact frog muscle was 2.54 and 1.09 m. eq. per 100 g. respectively, They assume that the excess sodium is contained inside the fibres. Mond and Netter (1932) had observed a similar difference and supposed that the sodium was bound on the surface of the fibres. On immersing the muscle in the Ringer's solution, the contents of both sodium and potassium in the muscle rise, but the chloride rises more than the sodium till the molar content of the two becomes equal (about 3.6 m. eq per 100 g.). It is difficult to account for these differences in the two conditions. It may be that the properties of membranes and the relative

sizes of the "cells" and the "interspaces" become changed when the muscle is immersed in the Ringer's solution. But without more precise knowledge it is not possible to form an idea of these changes, if any, that take place in the structure and composition of muscle when it is isolated and bathed in Ringer instead of lymph or serum.

III Possibility of an exchange of certain substances like phosphate, lactate and creatine between the "cells" and the "interspaces" under certain conditions. In the above discussion it has been shown that a number of substances are unable to diffuse through membranes between the "cells" and the "interspaces". It may now be considered if under certain conditions such an exchange is possible. In the case of some there is no evidence of such an exchange. Thus carnosine remains confined to the "cells" whether the muscle is fatigued in situ or in the Ringer solution. Similarly chloride appears to be unable to diffuse into the "cells" under these conditions. The case is different, however, for ions like phosphate, lactate and creatine. The evidence of the probability of lactate diffusing from the "cells" into the "interspaces" has been given elsewhere.[1] In the case of phosphate, M.G. Eggleton found that an increase in the inorganic phosphate in the "cells" caused by stimulation of the muscle, caused a corresponding rise of inorganic phosphate in the "interspaces". It has also been shown that an increase in the concentration of phosphate in muscle by exercise causes a definite though a small rise of the phosphate content of blood (Havard and Reay 1926) In the case of creatine, Eggleton (1930) found that the concentration of this substance in the Ringer required to prevent a loss or gain of creatine from the muscle

1 See p. 19.

rises and falls with a corresponding rise or fall in the total concentration of this substance in the muscle. It appears, then, that under certain circumstances an exchange of these ions between the "cells" and the "interspaces" is possible.

It has been frequently observed that the state of excitation causes increased permeability in cells. Thus Lillie (1911) has shown that yellow pigment contained in the cells of Arenicola leaves them when the cells become tonically contracted. It might have been supposed that a state of contraction or mere tone of muscle would allow an exchange of some ions between the "cells" and the "interspaces". Evidence in favour of such a hypothesis is not convincing. McClendon (1912, 1927) found an increase of electrical conductivity in muscle in an excited state, an index of the increased permeability of ions. Hartree (1933), on the other hand, did not detect any measurable effect on its electrical resistance on stimulation. It may be noted, however, that Hartree used a current of 100,000 cycles, while McClendon employed a low frequency current of 1,000 cycles, and the different results obtained by these writers may be due to this fact. Embden and Adler (1922) suggested that membranes become permeable to phosphate when the muscle is stimulated, as they found increased diffusion of phosphate from muscle into the surrounding saline. It is now known that

stimulation causes breakdown of phosphagen and therefore increases the total inorganic phosphate content of muscle. But as a large proportion of prophagen must be contained within the "cells", these experiments do suggest a diffusion of phosphate from the "cells" into the saline. Ernst and Csucs (1929) have, however, pointed out that the results of Embden and Adler are obtained only on direct stimulation of the muscle. On indirect stimulation there is no loss of phosphate from it. Since on indirect stimulation there is just the same breakdown of phosphagen, as on direct stimulation, (Nachmansohn, 1928) these authors conclude that phosphate diffuses out of muscle on direct stimulation, simply because the electric current damages the muscle. This conclusion is supported by the observations of Mond and Netter (1930) who find that muscle does not give up any potassium or take up chloride on indirect stimulation, although it does so when stimulated directly.

The writer, in the experiments already recorded, did not observe any change in the value of α for lactate when the muscles were stimulated during the course of the experiment. It may be concluded, therefore, that there is no direct evidence to suggest that a state of excitation of muscle allows these ions to diffuse through the membranes between the "cells" and the "interspaces".

It is possible that a change in the pH of muscle "cells" might change the properties of membranes so

that they allow certain ions to diffuse out. Fenn and Cobb (1934) have shown that one of the factors regulating the diffusion of potassium into or out of muscle is the pH of the saline, a rise in the pH of the saline increasing the diffusion of potassium into the muscle. Meyerhof (1921) observed that in the case of a muscle fatigued in a neutral Ringer solution only 5 p.c. of its lactic acid content diffused into the Ringer, while if the Ringer was strongly alkaline (pH 10), diffusion into the Ringer was as much as 20 p.c. And Clark, Gaddie and Stewart (1932) have shown that in perfused heart the excretion of lactic acid depends on the reaction of perfusion fluid. Excretion occurs freely at pH 8.5 and very slowly at pH 7.0.

The evidence presented here is, however, far from being conclusive to attribute the apparent diffusion of phosphate and lactate from the "cells" into the "interspaces" as being due to a change of pH in the muscle "cells", and it must be admitted that the mechanism by which this is accomplished in the intact animal remains unexplained.

IV

Histological site of the interfaces between the "cells" and the "interspaces."

In the above discussion it was clearly understood that the terms "cells" and "interspaces" had no histological significance and were used merely as convenient expressions of two fractions of muscle which appeared to be separated by some form of membrane which affected the diffusion of dissolved substances between them. An attempt to locate the site of this interface by histological methods is beset with difficulties, as distortion of tissue during preparation for sections is inevitable. An attempt by the writer to measure the relative areas of interspaces, muscle fibres and of areas of Conheim in transverse sections of voluntary muscle did not give very consistant results, and a detailed account of these observations is therefore omitted. But in the preparations which seemed to have undergone the least amount of distortion it was clear that the fractions "cells" and "interspaces" could hardly correspond to histological fibres and interspaces, as the latter (including connective tissue, blood vessels etc.) did not seem to form more than 8 - 10 p.c. of the total area of the section. It seemed, however, that the areas of Conheim did constitute about two thirds or a little more of the total area. These observations suggest that it is possible that the sarcostyles constitute the "cells" of the muscle and the interfaces between the sarcostyles and the sarcoplasm, the membranes regulating the diffusion

process. The matter, however, requires further careful investigation.

SUMMARY.

1. Diffusion of various substances into and out of voluntary muscle of frog as investigated by various authors is discussed from the standpoint of the present investigation. It is shown that

(i) There is evidence that carbon dioxide, oxygen urea, histidine, potassium and certain other substances can diffuse through the whole of muscle water.

(ii) A number of substances appear to be able to diffuse from the Ringer's solution into a fraction of muscle water ("interspace" water) but are unable to diffuse under these conditions in the rest of the muscle water (water in the "cells"). In all cases where the value of these two fractions can be calculated, the fraction of water in the "interspaces" (α) is found to be about one third of the total muscle water.

(iii) In an isolated resting muscle immersed in Ringer's solution, sodium, chloride and probably glucose appear to be confined to the "interspaces" only, none being present in the "cells". Added iodide and sulphate are also unable to diffuse into the "cells". On the other hand, carnosine and a large fraction of potassiumappear to be confined to the "cells", while phosphate, lactate, bicarbonate and creatine are present in both the fractions of muscle water, but under the conditions specified above, there appears to be no exchange

of these substance between these two fractions.

2. A table is appended giving the concentration of various dissolved substances in these two fractions of muscle water, calculated from the data available.
3. Adsorption does not seem to play a significant part in the distribution of these substances, nor is there any evidence of membrane equilibria of Donnan type, except in the case of potassium.
4. Variations in the value of α in different muscles in conditions of fatigue, in heat rigor, and on changing the composition of the immersing fluid are discussed.
5. Reasons are given for the probability of an exchange of certain substances like phosphate, lactate and creatine between the "cells" and the "interspaces" under certain conditions, especially in the intact animal. With regard to possible conditions allowing this exchange, an excited state of muscle and the effect of a change in the pH of the "cells" are discussed.
6. A preliminary attempt is made to locate the interface between the "cells" and the "interspaces" by histological methods. It is concluded that the fractions "cells" and "interspaces" could hardly correspond to the histological fibres and interspaces; but it is possible that the interfaces between the sarcostyles and sarcoplasm may constitute the membranes regulating the diffusion process.

APPENDIX A.

METHOD OF THE DETERMINATION OF LACTIC ACID.

Micromethods for the determination of lactic acid date from 1922 when Clausen developed the technique to estimate as little as 0.2 mg. of lactic acid. Clausen employed either 50 p.c. H_2SO_4 or 0.005 N potassium permanganate solution for the oxidation of lactic acid to acetaldehyde. The aldehyde thus formed is distilled over (with the assistance of a current of air), into an excess of sodium bisulphite solution to form acetaldehyde sodium bisulphite (Ripper 1900):

$CH_3.CHO + NaHSO_3 \rightleftharpoons CH_3.CHOH.SO_3.Na$, the excess of bisulphite is neutralised by 0.1 N iodine, the end point is adjusted to a definite blue colour using starch as indicator. The acetaldehyde bisulphite compound is now decomposed by rendering the solution alkaline with $NaHCO_3$ and the previously bound sulphite is titrated with accurately standardised .005 N iodine solution.

Friedemann, Cotonio and Shaffer (1927) modified Clausen's method by using manganous sulphate solution in sulphuric acid to catalyse the oxidation, introduced a reflux condenser to lessen the carriage of water into the receivers and accurately defined the conditions for titration. Friedemann and Kendall (1929) corroborated the fact that in the case of blood and muscle, the above method (where the interfering substances chiefly proteins and sugar can be efficiently removed) gives consistant results.

Eggleton, Eggleton and Hill (1928) used the Clausen's technique as modified by Friedemann, Cotonio and Shaffer, but without aeration, and found that the technique had (within the range of quantities of 0.2 to 1.5 mg. of lactic acid) an efficiency of 97½ p.c. as judged by the recovery of added lactate. This method was used for the estimation of lactic acid by the writer.

The apparatus consisted of a Kjeldahl flask with a delivery tube about one inch below the neck carrying a Kjeldahl trap. The flask was fitted with a glass stopper carrying a tap funnel from which N/200 $KMnO_4$ solution was dropped into the fluid in the flask.

The proteins were precipitated with 4 p.c. trichlor-acetic acid. In the case of Ringer, the acid was added to the saline to bring it up to a definite volume (usually 20 c.c.). In the case of muscle, the latter was rubbed up with a small quantity of ice-cold acid in a cooled morter, till it was reduced to a thin paste. More acid was added to bring it up to the desired volume. In either case the solution, after filtration was neutralised with caustic soda. The protein free filtrate was then treated with copper sulphate and lime to remove sugar and other interfering substances, as described by Van Slyke (1917).

An appropriate portion of the filtrate from the copper-lime treatment was then transferred to the Kjeldahl flask, together with 5 c.c. of acid

manganous sulphate solution and the solution was diluted with water to bring up the volume to 50 c.c. so that it may contain 1 p.c. of sulphuric acid. The flask was heated by a micro-burner, and $KMnO_4$ solution added rapidly but not in sufficient amount to produce a permanent pink colour in the solution. The vapours of acetaldehyde were passed through a double surface condenser, and collected in a receiver containing 5 c.c. of 2p.c. potassium bisulphite solution. Direct estimation of the bound metabisulphite was carried out with 0.005 N iodine (or 0.002 N iodine when the quantity of lactate estimated was small), using starch as indicator.

Blank determinations. An average of 0.2 c.c. of N/200 iodine was required for the blank to give the same iodine-starch reaction as the standard used in the lactic acid estimations. 0.2 c.c. of iodine was therefore subtracted from the amount of N/200 iodine required for all determinations of lactic acid. A blank determination was usually made with each experiment as a check to the reliability of the results obtained.

Recovery of lactate from aqeous solutions.

A solution of lithium lactate was prepared by dissolving 0.2 g. of the salt in 1 litre of water. 2 c.c. portions of the solution were taken and the amount of lactate present estimated as described above. An average of all the experiments showed a yield of 95.5 p.c. of the theoretical amount, with a variable error of 5 p.c.

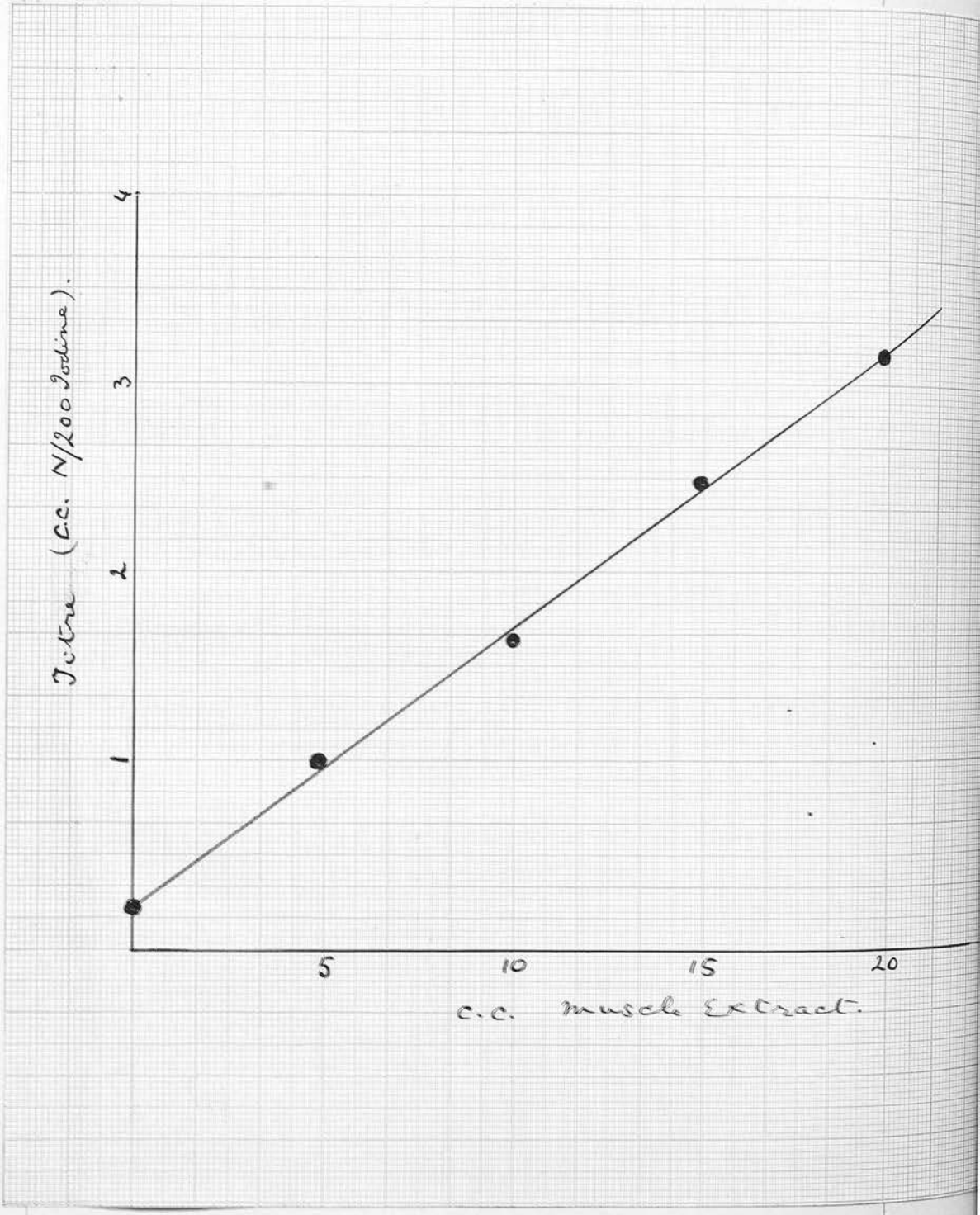

FIG. 9. Recovery of lactate from muscle extract.

Recovery of lactate from muscle extracts.

A number of experiments were performed to test the consistency of results of the estimations from varying amounts of muscle extracts. The general plan was to prepare a muscle extract with 4 p.c. trichlor-acetic acid, and take varying amounts of the extract, bring each portion to a definite volume with the acid, and estimate the lactic acid content of each. Thus in one experiment, the volumes of the extract taken, and trichlor-acetic acid added were as follows:

4 p.c. Trichloracetic acid	c.c.	20	15	10	5	0
Muscle Extract in 4 p.c. Trichloracetic acid	c.c.	0	5	10	15	20

The results of determination of this experiment are represented graphically in Fig. 8 ., in which the c.c. of muscle extract used are plotted against c.c. of N/200 iodine required to titrate the bound bisulphite. It will be seen that a good straight line results, indicating a consistant recovery in each case. Incidentally the line cuts the vertical axis at a point indicating a titre of 0.2 c.c. of iodine, which confirms the magnitude of the blank error of the method as obtained by direct estimation.

APPENDIX B.

Preparation of Zinc d-lactate.

1. Preparation of protein free muscle extract.

The technique described by Eggleton and Eggleton (1932) was applied. It depends on the fact that the solubility of sodium sulphate in water is at its maximum (55 gms. per 100 gms. of water) at 32°C., but at 0°C., 96 p.c. of the salt crystalises out as a decahydrate.

Butcher's meat, after being freed from fat and gristle was finely minced. Anhydrous sodium sulphate (400 gms. per kilo of muscle — calculated amount to form saturated solution in the water of the muscle at 32°C.) was then well mixed with the mince, and the whole incubated at 34°C overnight. It was then pressed out while still warm. The xtract was filtered and cooled under a tap to about 18°C., and crystals of sodium sulphate filtered off in a Buchner funnel. The filtrate was then cooled to 0°C. and the fresh crop of crystals formed was again separated. The mother liquor contains, besides other water soluble constituents of muscle, lactic acid in concentration about three times greater than the muscle.

The yield was increased by working up the residue as follows:

The pressed beef cake was broken up and mixed with (i) sodium sulphate crystals separated in the above process, and (ii) an amount of water equal to the amount of the filtrate separated; and incubated at 34°C. A protein free extract was again prepared from this mixture by repeating the process described above.

2. Extraction of lactic acid. The mother liquor was acidified with sulphuric acid, and extracted with ether, in a continuous extraction apparatus. The extraction is a slow process, 30 - 35 hours being required to remove the greater part of the acid (Embden (1912), Wolf (1914).

3. Preparation of Zinc d.-lactate. The ether extract was slowly evaporated to a small volume, dissolved in about 10 times its weight of hot water, and neutralised with zinc carbonate, by adding small quantities of carbonate at a time till further addition produced no effervescence. The solution was filtered hot. About 2 volumes of acetone were added and zinc lactate allowed to crystallise at 0°C. The crystals were redissolved in hot water, filtered, and re-precipitated with alcohol, the process being repeated twice, and finally the crystals were desiccated over sulphuric acid. After each recrystallisation the salt was examined under the microscope and polarimetrically. The final optical activity was as follows:-

$$[\alpha]_D^{19} \left(=\frac{100\theta}{cl}\right) = \frac{-0.87 \times 100}{5.01 \times 2.2} = -7.89.$$

θ is the observed angle of rotation, l is the length of the tube and c, the concentration of the solution.

The concentration was determined by estimation of the lactic acid content of the solution. 100 mg. of the solution was found to require 15.67 c.c. N/200 iodine. Estimation of the dry salt for its lactic acid content indicated that 1 mg. of the salt yielded

acetaldehyde equivalent to 3.10 c.c. N/ 200 iodine. 1 mg. of Zn $(C_3H_5O_3)_2$ should require 3.13 c.c. of N/200 I_2, allowing 4 p.c. loss in the distillation. The solution must therefore have contained 5.06 g. of the Salt per 100 g.

The optical rotatory power of Zinc d- lactate varies with the degree of ionisation of the salt and therefore increases on dilution. The value found above agrees fairly well with those of Purdie (1893) and Irwin (1906) who found values of -7.74 and -7.85 for concentrations of 6.4 and 6.18 p.c. of anhydrous salt respectively. The figures given by Hoppe-Seyler and Araki, as quoted by International critical Tables are somewhat lower. They give an $[\alpha]_D^{15} =$ -0.6 for a concentration of 5 p.c., and -0.82 for one of 2.5 p.c.

APPENDIX C

Time required for diffusion equilibrium to be attained between muscle and Ringer's Solution with regard to lactate.

The following procedure was adopted. Hind limb muscles of several frogs (R. esculenta (Hungary)) were dissected and 50 g. of the muscles were immersed in 50 c.c. of modified Ringer's solution containing about 400 mg. of lactate per 100 g. Oxygen was bubbled through continuously to maintain gentle stirring, and the beaker was kept at 0^{o} c. by packing it with ice. Another lot of 50 g. of muscles was similarly treated but nitrogen bubbled through instead of oxygen. 1 c.c. portions of the Ringer were removed at intervals varying from $\frac{1}{2}$ hour to 24 hours from the time of immersion for the estimation of lactic acid. Details are given in Table 6, (Experiments 1 and 2). It will be seen that the lactate content of the Ringer becomes practically constant and therefore diffusion equilibrium is attained between 2 - 4 hours. The equilibrium time was more accurately determined between the periods 2 - 4 hours in another experiment (Table 6, Experiment 3), which showed that the time required is 3 - $3\frac{1}{4}$ hours. From these experiments it was concluded that 4 hours was a safe period to allow lactate equilibrium to be attained between the Ringer and muscle in the experiments described in the text.

TABLE 6. Time required for diffusion equilibrium between Ringer's solution and muscles with regard to lactate.

Period during which diffusion was allowed to proceed HOURS.	Finalconcentration of the Ringer solution. (Mg. per c.c.) EXPERIMENT 1.	Final concentration of the Ringer's solution (Mg. per c.c.) Experiment 2.
0	3.70	3.70
½	3.54	3.53
1	3.37	3.45
2	3.20	3.31
4	3.00	3.20
8	3.12	3.24
24	3.13	3.21

Period during which diffusion was allowed to proceed HOURS	Final concentration of the Ringer's solution. (Mg. per c.c.) EXPERIMENT 3.
0	3.65
2	3.42
2¼	3.41
2½	3.37
2¾	3.36
3	3.31
3¼	3.28
3½	3.29
3¾	3.26
4	3.30

APPENDIX D.

Water content of muscles in heat rigor.

In the diffusion experiments on muscles in heat rigor, the muscles after being incubated at 40°C for 5 hours, were immersed in a Ringer's solution for about 16 hours before starting the diffusion experiment. By this treatment the muscles are bound to lose some proteins and gain some water by imbibition, so that their water content may be expected to be more than fresh muscles. It became necessary, therefore, to calculate the water content of such muscles in order to calculate the value of α. for this purpose the muscles were first sent into heat rigor and given a preliminary immersion as above described. They were then weighed, and dried in an oven at 105 -110°C to constant weight (12 - 16 hours). Table 7 gives the weights of wet and dry muscles. The solids in the muscles in heat rigor amount to about 17 p.c. of the moist weight of the muscles in Rigor after the preliminary soaking. The water content of these muscles is therefore 83 p.c. of the total moist weight.

TABLE 7 . Water content of frog muscles in heat rigor.

	Weight of muscle (g). (Fresh)	Weight of muscle in rigor (g)	Weight of muscle in rigor after soaking in Ringer solution. (g)	Dry weight of muscle. (g)	Percentage of water of muscles in rigor after soaking. (calculated)
1	-	1.171	1.685	0.1913	83
2	-	1.180	1.2375	0.2336	82
3	1.660	1.458	1.5445	0.2515	84
4	1.505	1.316	1.4039	0.2094	84
5	1.9925	1.708	1.805	0.3410	82
6	2.2585	1.950	2.0341	0.3736	82

TABLE 8 . Diffusion of d-lactate into and out of Resting Skeletal muscle of Frog.

		Weight of muscle. Gms. Initial (1)	Final (2)	Lactate Content of muscles. Mgms. Final (3)	Weight of Lactate Ringer. Gms. Initial (4)	Final (5)	Lactate Content of Ringer. Mgms. Initial (6)	Final (7)	Proportion of Muscle water concerned in Diffusion (P.C.) (8)
1	Lo	2.18	2.186	0.187	1.854	1.648	0	0.159	38
	L	2.254	2.264	0.420	2.106	1.822	1.74	1.40	
2	Lo	2.018	1.914	0.1858	2.778	2.730	0	0.1826	32
	L	2.034	1.986	0.3020	2.589	2.444	0.8465	0.8422	
3	Lo	1.7135	1.7165	0.1801	1.5427	1.4733	0	0.1341	32
	L	1.7165	1.7158	0.3490	1.5264	1.1454	1.0018	0.8668	
4	Lo	1.619	1.620	0.1745	2.1882	2.0882	0	0.1775	42
	L	1.610	1.6425	0.2813	2.3314	2.2079	1.438	1.327	
5	Lo	1.061	1.062	0.163	2.107	2.037	0	0.105	27
	L	1.153	1.143	0.213	2.132	2.079	1.834	1.760	
6	Lo	1.04	1.054	0.209	1.979	1.801	0	0.123	38
	L	1.061	1.055	0.878	2.005	1.936	7.714	6.736	
7	Lo	2.108	2.148	0.196	1.674	1.506	0	0.103	33
	L	2.162	2.122	0.591	1.824	1.580	3.374	2.79	
8	Lo	1.082	1.127	0.640	2.592	2.473	0	0.440	45
	L	1.130	1.110	1.80	2.528	2.476	8.25	7.42	

Lo = "Low lactate" Ringer. L = "High lactate" Ringer.

Experiments 1 - 7 were performed on previously cooled frogs, the rest on frogs from the tanks.

Table continued on next page.

TABLE 8 . (Contd.) Diffusion of d-lactate into and out of Resting skeletal muscle of Frog.

		Weight of muscle. Gms. Initial (1)	Final (2)	Lactate Content of muscles Mgms. Final (3)	Weight of Lactate Ringer. Gms. Initial (4)	Final (5)	Lactate Content of Ringer. Mgms. Initial (6)	Final (7)	Proportion of Muscle water concerned in Diffusion. (P.C.) (8)
9	Lo	0.811	0.836	0.780	0.708	0.614	0	0.360	28
	L	0.833	0.794	1.12	0.504	0.487	1.59	1.50	
10	Lo	1.189	1.245	0.87	0.602	0.588	0	0.250	48
	L	1.226	1.168	1.15	0.763	0.744	2.45	1.73	
11	Lo	1.027	1.045	1.01	0.613	0.529	0	0.380	32
	L	1.033	0.991	1.64	0.665	0.632	3.07	2.43	
12	Lo	1.047	1.076	1.11	0.897	0.787	0	0.50	39
	L	1.058	1.061	2.15	0.747	0.671	3.39	2.61	
13	Lo	1.410	1.433	1.16	1.220	1.083	0	0.50	41
	L	1.381	1.399	2.09	1.144	1.065	3.72	2.96	
14	Lo	2.104	2.145	0.460	1.547	1.406	0	0.18	34
	L	2.025	1.988	1.36	1.255	1.193	3.34	2.99	
15	Lo	1.059	1.070	0.47	1.061	0.999	0	0.260	34
	L	1.095	1.092	1.160	1.118	1.053	3.03	2.66	
16	Lo	1.545	1.588	0.44	1.203	1.070	0	0.301	32
	L	1.516	1.537	1.41	1.192	1.079	3.81	2.87	

Experiments 9 - 16 were performed on frogs fresh from the tank.

Lo = "Low lactate" Ringer. L = "High lactate" Ringer.

TABLE 9. Diffusion of lactate into and out of muscle in heat rigor.

	Wt. of muscle g.	Lactate content of muscles finally mg.	Wt. of Ringer solution g.	Lactate content of Ringer mg.		Concentration of lactate of preliminary soaking mg. per 100 c.c.	Proposition of muscle water involved in diffusion ( ) p.c. Calculated
				Initially	Finally		
1.	1.179	2.354	2.261	4.228	5.042	305	101
2.	1.127	4.035	1.637	8.137	7.015	305	92
3.	1.184	1.83	1.653	1.967	3.439	362	97
4.	1.695	3.592	1.865	8.222	7.775	363	83
5.	0.808	1.317	1.534	2.328	2.982	315	81
6.	0.9255	1.865	1.569	2.380	3.384	388	76
7.	0.9265	2.831	1.819	8.262	7.949	388	78

TABLE 10. Diffusion of lactate into and out of fatigued muscles of frog.

	Weight of muscle (g.)		Lactate Content of muscle Mg.	Weight of lactate Ringer (g.)		Lactate Content of Ringer Mg.		Proportion of muscle water (α) involved in diffusion. p.c.
	Initial	Final	Final	Initial	Final	Initial	Final	
1 Lo	0.416	0.3865	0.704	0.6535	0.615	0	0.229	7
L	0.451	0.426	0.8187	0.6705	0.608	2.179	2.30	
2 Lo	1.030	1.100	2.831	1.180	1.045	0	0.502	9
L	1.073	1.069	3.199	1.150	1.085	3 .381	3.669	
3 Lo	0.8845	0.948	1.611	0.8325	0.6810	0	0.294	-3
L	0.8995	0.8855	1.620	0.6545	0.5715	1.924	2.317	
4 Lo	0.940	1.005	1.68	1.173	1.0295	0	0.3718	2
L	0.944	0.917	1.872	1.074	1.016	3.157	3.479	

Lo = "Low lactate Ringer".

L = "High lactate Ringer".

TABLE 11. Diffusion of lactate into and out of isolated muscles, stimulated with single induction shocks (experiments 1 and 2) or short tetani (experiment 3).

		Weight of Muscle. G.		Lactate content of muscle. Mg.	Weight of Lactate Ringer. (g)		Lactate Content of Ringer. (Mg.)		Proportion of Muscle water (α) involved in diffusion.
		Initial	Final	Final.	Initial	Final	Initial	Final	p.c.
1	Lo	1.061	1.062	0.163	2.107	2.037	0	0.105	27
	L	1.153	1.143	0.213	2.132	2.079	1.834	1.760	
2	Lo	1.04	1.054	0.209	1.979	1.801	0	0.123	38
	L	1.061	1.055	0.878	2.005	1.936	7.714	6.736	
3	Lo	2.108	2.148	0.196	1.674	1.506	0	0.103	33
	L	2.162	2.122	0.591	1.824	1.580	3.374	2.79	

Lo = "Low lactate Ringer" L = "High lactate Ringer".

Frogs were cooled overnight at 2° C. and the experiments performed at 7°C.

APPENDIX F.

Estimation of the Iodide Content of Muscle.

The muscle is rubbed up with freshly prepared tungstic acid and brought up to a definite volume. The proportion of the acid and water used per gram of muscle is the same as advised by Folin and Wu (1919). It is then filtered with a Buchner funnel after the addition of a little kiesulghur. Keisulghur greatly lessens the tendency to form foam when the filtrate is subsequently shaken with chloroform. The filtrate is then measured and transferred to a separating funnel. Iodine is liberated from iodide by adding nitrous acid [1] (prepared by adding 3 drops of 5 p.c. sodium nitrite and 3 drops of 5 p.c. nitric acid to the solution), and repeatedly extracted with small quantities of chloroform. The chloroform is then titrated against N/500 or N/1000 sodium thiosulphate solution, after the addition of a few crystals of potassium iodide and two drops of starch solution.

Recovery of iodide by this method was tested by adding definite volumes of N/100 potassium iodide solution to muscle, and after the above treatment, estimating its iodide content. The results of one of these experiments are given in Table 12. The recovery of iodine is about 99 p.c. of the theoretical amount, with a variable error of about 5 p.c.

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1. In some estimations a saturated solution of $KMnO_4$ was used instead of nitrous acid to liberate iodine.

TABLE 12.

c.c. of N/100 KI added to muscle.	c.c. of N/500 thio-sulphate required for titration.
½	2.45
1	5.04
1	4.89
1½	7.48
2	9.85
2	9.88

APPENDIX G.

Time required for diffusion equilibrium to be attained Between Ringer's solution and muscle with respect to iodide.

About 50 g. of frog muscles were added to 50 c.c. of modified Ringer's solution containing sodium iodide in M/100 concentration. Gentle stirring was maintained by bubbling compressed air through the solution. one c.c. portions of the Ringer were removed at intervals of 0 hours to 20 hours from the time of immersion, and its iodide content estimated. The results of one of the experiments are given in Table 13 .

It will be seen that equilibrium is attained in about 5 hours.

TABLE 13.

Period during which diffusion was allowed to proceed. (hours)	c.c. N/500 thiosulphate per C.C. of Ringer required for titration.
0	5.15
1	4.71
2	4.39
4	4.32
5	4.25
6	4.21
8	4.28
20	4.23

TABLE 14. Diffusion of iodide into isolated fresh frog muscles.

	Weight of iodide Ringer (g) (Initial)	Iodide content of Ringer		Weight of Muscle (g)	Iodide content of muscle	Proportion of muscle water (α) involved in diffusion
		Initial	Final		Final	
1	1.471	3.06	2.677	1.267	0.36	21
2	1.669	3.47	2.971	1.263	0.51	31
3	1.317	3.13	2.75	0.974	-	23
4	1.141	2.71	2.35	0.953	-	22
5	1.104	1.513	1.18	1.403	-	28
6	1.314	0.626	0.534	1.376	-	21
7	1.652	5.19	4.18	1.886	0.97	25.5
8	1.618	3.535	2.83	1.942	0.66	23
9	1.960	9.048	7.616	1.217	1.40	37
10	1.969	3.82	3.39	1.213	0.40	28
11	1.960	4.467	3.41	2.285	-	31
12	1.852	2.717	1.918	2.037	-	41

TABLE 15. Diffusion of iodide into fresh thigh preparations of frog.

	Quantity of iodide Ringer. (Initial) c.c.	Concentration of iodide in Ringer (units per c.c.) Initial	Final	Weight of the thigh preparation (g)	Proportion of muscle water (α) involved in diffusion p.c. (calculated)
1	12	4.25	3.924	10.62	35
2	12	4.25	3.888	10.132	36
3	12	4.25	3.690	12.463	28
4	12	1.11	1.026	12.867	34
5	12	1.11	1.056	12.760	31
6	12	1.11	0.972	13.406	41

TABLE 16. Diffusion of iodide into isolated muscles, previously sent into heat rigor.

	Weight of iodide Ringer (g) (Initial)	Iodide content of Ringer		Weight of muscle (g)	Proportion of muscle water (α) involved in diffusion.
		Initial	Final		
1	1.652	1.991	1.253	1.236	92
2	1.941	7.602	4.488	1.622	102
3	1.481	10.788	6.842	1.135	95
4	1.672	3.903	2.207	1.486	103
5	1.522	9.122	5.667	1.172	95
6	1.735	3.286	1.920	1.547	96

TABLE 17. Diffusion of iodide into thigh preparations previously sent into heat rigor.

	Quantity of iodide Ringer Initial c.c.	Concentrations of iodide in Ringer (units per c.c.)		Weight of the thigh preparation. g.	Proportion of muscle water (α) involved in diffusion.
		Initial	Final		
1	15	4.25	3.24	10.924	101
2	15	4.25	3.492	10.745	80
3	15	4.25	3.450	10.71	84
4	15	1.11	0.864	10.46	97
5	15	1.11	0.876	9.006	108
6	15	1.11	0.852	11.034	98

In tables 14-17 the iodide contents are given in arbitrary units (= c.c. of N/500 sodium thiosulphate solution required for titration of iodine).

TABLE 18. The coefficient of diffusion, k, of iodide through muscle.

	c (Mg. per c.c.)	a (sq. cm.)	t (Minutes)	Amount Diffusing (MG.)	k
F	7.62	25.75	5	4.9	0.98×10^{-4}
			10	12.46	1.01×10^{-4}
			15	15.2	1.0×10^{-4}
F	5.289	25.5	½	1.26	1.37×10^{-4}
			3	2.46	0.87×10^{-4}
			5	3.39	1.0×10^{-4}
F	762	25.0	5	5.2	1.17×10^{-4}
			10	7.39	1.19×10^{-4}
F	4.992	25.19	1	1.548	1.19×10^{-4}
			4	2.682	0.90×10^{-4}
			6	3.387	0.95×10^{-4}
R	4.70	25.64	1/6	1.785	10.2×10^{-4}
			½	2.94	9.3×10^{-4}
			1	3.765	9.1×10^{-4}
R	5.23	25.00	1/6	1.771	8.64×10^{-4}
			½	3.032	8.46×10^{-4}
			1	4.308	8.51×10^{-4}
R	5.175	25.46	1/6	1.65	7.4×10.4
			5/12	2.655	7.6×10^{-4}
			¾	3.469	7.3×10^{-4}
R	4.56	26.82	1/6	1.678	8.88×10^{-4}
			½	2.867	8.64×10^{-4}
			1	4.045	8.64×10^{-4}

F = Fresh muscles. R. = Muscles in heat rigor.
For explaination of the terms c, a, t and k, see text.

TABLE 19. The coefficient of diffusion, k, of iodide through agar jelly.

c (Mg. per c.c.)	a (Sq. cm.)	t (Minutes)	Total amount diffusing (Mg.)	k.
9	4.91	5 12.5 22.5	3.135 5.283 6.960	8.7 X 10-4
9	4.91	5 10 15	3.336 4.839 5.727	9.1 X 10-4
9	4.91	3 8	2.45 4.12	8.4 X 10-4
9	4.91	9 36 81 144	4.428 4.565 4.534 4.588	9.4 X 10-4

For explaination of the terms c, a, t and k, see text.

APPENDIX J.

Determination of the total CO_2 content of muscle.

The muscle is removed from the bicarbonate Ringer rapidly blotted with moist filter paper, and immersed into 1 c.c. of 0.5 p.c. CO_2 free caustic soda solution [1] to fix the dissolved CO_2. 0.5 c.c. of 5 p.c. H_2SO_4 is then added to the solution to render it acid, and the tube fitted with a perforated rubber stopper and a delivery tube. The other end of the delivery tube dips into 1 c.c. of 0.5 p.c. NaOH solution contained in a short test tube. The tube containing the muscle is heated with a microburner, and the solution boiled for 5 minutes to drive out all CO_2, which passes out by the delivery tube and is trapped in the caustic soda solution. The tube containing the caustic soda solution is then removed and boiling stopped. The contents of the tube are emptied into the cup of Van Slyke's apparatus and then drawn into its chamber by cautiously opening the stop cock leading to it. The test tube is washed out with small amounts of water and the washings transferred to the cup and then into the chamber, till 2.5 c.c. of fluid has been drawn into the chamber. Lastly 0.5 c.c. of 5 p.c. H_2SO_4 are introduced into

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1. This solution is prepared and kept as advised by Van Slyke (1927, a)

the chamber. Estimations of the total CO_2 is then performed in the usual way. A blank estimation is performed, and the volume of gas obtained in the blank is subtracted from the total volume of gas determined.

Estimation of the CO_2 content of twin gastrocnemii directly removed from a frog may give a difference of about 10 p.c. by this method. The method is not yet fully standardised, and may require some modification in later work. It may be mentioned in passing that the estimation of the CO_2 content of muscle has not been used for the estimation of the value of α in the present work, but only for determining the relative concentration of bicarbonate in the "cells" and the "interspaces."

TABLE 20. Diffusion of bicarbonate.

	Wt. of Muscle (g)	Bicarbonate content of muscle. finally (c.c.)	Volume of Ringer	Bicarbonate content of Ringer (c.c.) Initially	Finally	Proportion of muscle water ( ) in diffusion process. (calculated)	Concentration of bicarbonate. (calculated) (c.c. per 100 g.) in "inter-spaces" of muscle.	in "cells" of muscle.	pH of Ringer finally (calculat
1. Lo	0.948	0.131	1.0	0	0.144	35	68	18	6.32
L	0.948	0.280	1.0	0.726	0.7164		67	17	7.02
2. Lo	0.6465	0.1053	1.0	0	0.090	40	52	19	6.12
L	0.6435	0.259	1.0	0.686	0.666		57	26	7.00
3. Lo	0.665	...	1.5	0	0.1314	43	..	..	6.11
L	0.675	...	1.5	1.088	10.73		..	..	7.02
4. Lo	0.826	0.134	1.5	0	.047	39	24	18	6.04
L	0.822	0.235	1.5	1.088	.9831		26	15	6.98
5. Lo	0.950	...	2.0	0.251	0.302	34	..	..	6.42
L	0.955	...	2.0	0.964	0.938		..	..	6.91
6. Lo	1.088	...	2.0	0.51	0.49	23	..	..	6.62
L	1.089	...	2.0	1.12	1.08		..	..	6.97
7. Lo	1.00	...	2.0	0.781	0.65	33	..	..	6.95
L	0.9961	...	2.0	1.354	1.202		..	..	7.29

Lo = "Low bicarbonate" Ringer. L = "High bicarbonate" Ringer.
Experiments 1 - 3 performed at 0°C. but CO_2 estimations at room temperature.
Experiments 3 - 7 performed and estimations carried out at 7°C. in a cold room.

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